

**The Control of Decidual Prolactin Production
during Human Pregnancy**

Wen Xuan Wu B.M. & M.M.

**Doctor of Philosophy
Medical School, University of Edinburgh
October 1991**



CONTENTS

	Page
Declaration	i
Acknowledgements	ii
Abstract	iii
List of Abbreviations	v
List of Figures and Tables	vi
List of Plates	viii
 CHAPTER 1: The Background of this Thesis	
1.1. Introduction	1
1.2 Human Endometrium and Decidualization	1
1.2.1. The Cellular Elements in Human Endometrium	1
1.2.2. The Subcellular Sites of Action of Progesterone and Oestrogen	2
1.2.3. The Regulation of Oestrogen and Progesterone Receptors	5
1.2.4. Decidualization	7
1.3. Identification of Decidual Prolactin	12
1.4. The Source of Decidual Prolactin	12
1.5. The Biochemical Fate of Decidual Prolactin	14
1.6. The Regulation of Synthesis and Release of Decidual Prolactin	16
1.6.1. Dopamine and Thyrotropin-Releasing Hormone	16
1.6.2. Oestrogen and Progesterone	16
1.6.3. Factors Derived from the Utero-Placental Unit	18
1.6.4. Intracellular Signalling System	19
1.7. The Physiological Function of Decidual Prolactin	20
1.7.1. The Localization of Prolactin Receptor in the Utero-Placental Unit	20
1.7.2. The Effect of Decidual Prolactin on Water and Minerals across the Fetal Membrane	21

1.7.3. The Effect of Decidual Prolactin on Fetal Lung Maturation	22
1.7.4. Other Effects of Decidual Prolactin	22
1.8. Decidual Prolactin Gene Expression	23
1.9. The Aims of this Thesis	25

CHAPTER 2: Localization of the Sites of Synthesis and Action of Prolactin by Immunocytochemistry and In Situ Hybridization within the Human Utero-Placental Unit

Part I. Development of an In Situ Hybridization Method to Localize Prolactin mRNA in the Utero-Placental Unit

2.1.1. Introduction	27
2.1.2. Materials and Methods	28
2.1.3. Results	31
2.1.4. Discussion	32

Part II. Modification of the Immunostaining Method for Immunolocalization of Decidual Prolactin in Utero-Placental Unit

2.2.1. Introduction	38
2.2.2. Materials and Methods	38
2.2.3. Results	40
2.2.4. Discussion	40

Part III. Localization of the Sites of Synthesis and Action of Prolactin by Immunocytochemistry and In Situ Hybridization within the Human Utero-Placental Unit

2.3.1. Introduction	45
2.3.2. Materials and Methods	46
2.3.3. Results	48
2.3.4. Discussion	52

CHAPTER 3: The Relationship between the Decidualization and Prolactin Production at the Different Stages of Human Pregnancy

6.1. Introduction

3.1. Introduction	55
3.2. Materials and Methods	55
3.3. Results	58
3.4. Discussion	63

CHAPTER 4: Sex Steroid Modulation of Prolactin Production by Human Early and Term Decidua

Part I. The Effects of the Anti Progestin, Mifepristone, In Vivo, and Progesterone in Vitro on Prolactin Production by the Human Decidua in Early Pregnancy

7.1.1. Introduction

4.1.1. Introduction	72
4.1.2. Materials and Methods	72
4.1.3. Results	76
4.1.4. Discussion	

Part II. Deposition of Prolactin in Decidual Cells Produced with mifepristone

Part II. The Combined Effect of Progesterone and Oestrogen on Prolactin Production by Human Term Decidual Cells

7.2.1. Introduction

4.2.1. Introduction	85
4.2.2. Materials and Methods	86
4.2.3. Results	92
4.2.4. Discussion	92

CHAPTER 5: Immunolocalization of Oestrogen and

Progesterone Receptors in the Human Decidua in Relation to Prolactin Production

8.1. The Effect of Oestrogen and Progesterone on Prolactin Production

5.1. Introduction	98
5.2. Materials and Methods	99
5.3. Results	104
5.4. Discussion	109

CHAPTER 6: Local Control of Decidual Prolactin Production by Non-Steroid Factors

6.1.	Introduction	114
6.2.	Materials and Methods	114
6.3.	Results	117
6.4.	Discussion	125

CHAPTER 7: Effect of Dopamine on Decidual Prolactin Production before and after Transfection of Decidual Cells with Dopamine D2 Receptor

Part I. Localization of Dopamine D2 Receptor Gene Expression in Human Decidua

7.1.1.	Introduction	129
7.1.2.	Materials and Methods	130
7.1.3.	Results	139
7.1.4.	Discussion	148

Part II. Dopamine Effect on Decidual Cells Transfected with Dopamine D2 Receptor

7.2.1.	Introduction	151
7.2.2.	Materials and Methods	151
7.2.3.	Results	153
7.2.4.	Discussion	153

CHAPTER 8: General Discussion

8.1.	Identification of the Cellular Origin of Decidual Prolactin	157
8.2.	The Effect of Oestrogen and Progesterone on Decidual Prolactin Production	159
8.3.	The Effect of Non-Steroid Factors and Dopamine on Decidual Prolactin Production	165

8.4. The Comparison of the Regulatory Systems Involved in the Synthesis and Release of Decidual and Pituitary Prolactin	168
---	-----

APPENDICES	171
-------------------	-----

BIBLIOGRAPHY	183
---------------------	-----

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree. This thesis was written by myself.

Wen Shan Wu

Department of Physiology

Acknowledgements

I am deeply indebted to Dr. A. S. McNeilly for his excellent supervision, encouragement, great guidance of my subject research and also for his putting up with my mistakes throughout my study, which was funded by the British Council and the State Education Commission of People's Republic of China. I wish to thank Dr. A. Oliver for his encouragement and organization of human studies for my study. Similarly, I wish to thank Dr. R. Kelly for teaching me basic knowledge.

DECLARATION

Except where acknowledgement is made by reference, the experiments detailed in this thesis were the unaided work of the author. No part of this work has previously been accepted for any other degree, nor is any part of it being concurrently submitted in candidature for another degree. This thesis was written by myself.

I wish to thank Dr. J. Brooke and Mrs. W. Taylor for their teaching me the basic laboratory work and their encouragement. I also wish to thank Mrs. P. Holland and Mrs. S. Eames for their friendly help in learning the basic skills of laboratory work in my first year. I wish to thank Dr. R. Kelly, Mr. Taylor and Mrs. J. Dehnbach for teaching me how to do a real molecular biologist.

Wen Xuan Wu

Thanks are also due to Dr. J. Norman, Dr. W. Ledger, and all the sisters in the labour ward and 36 and 38 wards for their collecting proper tissues for my study. I would like to acknowledge Mr. T. McFeters and Mr. E. Pinner for their taking photos and teaching me how to draw the graphs, and the staffs in the MRC Reproductive Biology Unit and postgraduate students for their help and friendship during the period of my study.

I would like to thank Prof. D. Martin for his accepting me as a graduate student in this unit.

Finally, I wish to thank Mr. and Mrs. Day and my husband D. for their constant encouragement and love throughout my study.

Acknowledgements

I am deeply indebted to Dr A. S. McNeilly for his excellent supervision, encouragement, great guidance of my English writing and also for his putting up with my 'east mind', throughout my study, which was funded by the British Council and the State Education Commission of People's Republic of China. I would like to thank Dr. A. Glasier for her encouragement and organization of human tissue for my study. Similarly, I wish to thank Dr. R. Kelly for teaching me basic knowledge of science and skills in the laboratory in the first year of my study.

I am grateful to Mr. M. Millar for his skilled help in the basics of histology, for his working with me to set up immunocytochemistry and in situ hybridization, and for his teaching me how to speak Scottish! I wish to thank Dr. J. Brooks and Ms. W. Crow for their teaching me the basic laboratory work and their friendship. I also wish to thank Ms. P. Holland and Ms. S. Lamda for their friendship and teaching me the basic skills of laboratory work in my first year study and thank to Dr. K. Eidne, Mr. Taylor and Ms. J. Zabavnik for teaching me how to become a real molecular biologist!

Thanks are also due to Dr. J. Norman, Dr. W. Ledger, and all the sisters in the labour ward and 35 and 36 wards for their collecting proper tissues for my study. I would like to acknowledge Mr. T. McFetters and Mr. E. Pinner for their taking photos and teaching me how to draw the graphs, and the staffs in the MRC Reproductive Biology Unit and postgraduate students for their help and friendship during the period of my study.

I would like to thank Prof. D. Lincoln for his accepting me as a graduate student in this unit.

Finally, I wish to thank Mum and Dad and my husband Qi for their constant encouragement and love throughout my study.

ABSTRACT

Decidual prolactin (PRL) is identical to pituitary PRL according to its chemical, biological, and immunological properties. The factors involved in the regulation of PRL production from pituitary and decidua, however, are different. Although much is known about the factors that modulate the pituitary production of PRL, very little is known about the control of decidual PRL synthesis and release. The studies in this thesis were undertaken to investigate the control of decidual PRL production during human pregnancy.

When this study was carried out, the exact cellular origin of decidual PRL remained unclear. Using the *in situ* hybridization with a human pituitary PRL cDNA probe, validated by Northern blot analysis, together with immunocytochemistry to co-localize PRL mRNA and its protein product, decidual cells in the utero-placental unit were identified conclusively, for the first time, as the only source of PRL synthesis.

As the decidual cells are one of the main targets of progesterone and estradiol action within the uterus, studies were carried out to examine the effect of these steroids on decidual PRL production. Firstly, the relationship between decidualization and PRL production at different stages of human pregnancy was examined using *in situ* hybridization and Northern blot to assess PRL mRNA level, immunocytochemistry to examine the PRL content inside the decidual cells and radioimmunoassay to measure PRL output in amniotic fluid. The results clearly show that PRL gene expression in human decidua paralleled the degree of decidualization which was resulted from the action of sex steroids. Further studies were performed to reveal how important the effect of progesterone on decidual PRL production was during early pregnancy by blocking the action of progesterone with the antiprogesterone (RU486) *in vivo*. Withdrawal of progesterone action *in vivo* only resulted in suppression of decidual PRL production associated with morphological de-decidualization in decidua parietalis free of trophoblast, while no such effect occurred in decidua capsularis which had trophoblast attached. The results indicated that decidual PRL production was not only dependent on progesterone, but also potentially on factors derived from the trophoblast. Finally, the effects of both oestrogen and progesterone, either alone or in combination on PRL

production by human term decidual cells, were investigated. This study clearly demonstrated that oestrogen and progesterone acted synergistically to maintain decidualization and consequent production of PRL by decidual cells which was further confirmed by studies of the immunolocalization of oestradiol and progesterone receptor. Progesterone induced and maintained PRL production via its receptor mechanism resulting in decidualization. Oestrogen modulated progesterone function at the progesterone receptor level, thus being indirectly involved in modulating PRL production.

Because the blastocyst-induced decidualization probably augments that initiated by sex steroids in human pregnancy, the potential PRL releasing activity associated with placenta was explored by adding placenta-conditioned medium and hCG, one of the major placenta proteins, to the cultured decidual. The results supported the concept that a local regulatory mechanism for decidual PRL production might exist which was related to placenta, but not hCG. The adenyl cyclase system (abcAMP) failed to effect, while TPA, testing the DAG pathway, inhibited decidual PRL production by term decidual cells.

Unlike pituitary PRL, dopamine had no effect on decidual PRL production. In situ hybridization appeared to indicate the presence of dopamine D2 receptors on decidual cells but Northern analysis and PCR of decidual mRNA with primers to both rat and human dopamine D2 receptors failed to confirm the presence of dopamine D2 receptors. Decidual cells transfected with the dopamine D2 construct also failed to response to dopamine (bromocriptin) suggesting the second messenger system associated with signal transduction of dopamine D2 receptor may not be functional.

These results support the concept that decidual cells are the only source of PRL in the utero-placental unit. The major control of release is the rate synthesis of PRL and this is related to the degree and maintenance of decidualization, induced and maintained by a synergistic action of oestradiol and progesterone. Dopamine is not a controller of decidual PRL production.

List of Abbreviations

DAB	3,3'-diaminobenzidine tetrahydrochloride	11
DAG	diacylglycerol	29
dbcAMP	dibutyryl cAMP	38
ER	oestrogen receptor	48
hCG	human chorionic gonadotropin	51
Ins P3	inositol triphosphate	71
IRMA	immunoradiometric assay	71
MOPS	3-[N-morpholino] propanesulphonie acid	77
PAF	picric acid-formaldehyde	77
PAP	peroxidase anti-peroxidase	78
PBS	phosphate buffered saline	78
PCR	polymerase chain reaction	78
PR	progesterone receptor	78
PRL	prolactin	81
SDS	sodium dodecyl sulphate	81
TBS	tris buffered saline	81
TPA	12-o-tetradecanoylphorbol-13-acetate	81

Fig. 4.5.	PRL production released by cultured term decidua cells treated with or without oestradiol and progesterone, either alone or in various combinations.	93
Fig. 5.1.	Comparison of progesterone production (A) and PRL production (B) in decidua parietalis and decidua capsularis in vitro during a 5 day incubation period.	109
Fig. 6.1.	The effect of PCM on the release of decidua PRL from early pregnancy decidua.	116
Fig. 6.2.	The effect of PCM on the release of decidua PRL from early pregnancy decidua.	119
Fig. 6.3.	The effect of PCM on the release of PRL from early and term pregnancy decidua.	121
Fig. 6.4.	The effect of dbcAMP on the release of PRL from term decidua cells.	122

List of Figures and Tables

	Page
Table 1.1. Human endometrium proteins.	11
Fig. 3.1. The quantitative results of in situ localization of PRL mRNA in early, mid and term decidua.	64
Fig. 3.2. The relationship between the decidual cell size and the level of PRL mRNA in early, mid and term decidua.	65
Fig. 3.3. PRL concentrations in the amniotic fluid in early, mid and term pregnancy.	66
Fig. 4.1. Production of PRL by decidua parietalis in vitro during a 5 day incubation period.	77
Fig. 4.2. Production of PRL by decidua capsularis in vitro during a 5 day incubation period.	78
Fig. 4.3. Comparison of PRL production by decidua parietalis and capsularis in vitro during a 5 day incubation period.	78
Fig. 4.4. Comparison of the area of decidual cells in sections (HE stain) of decidua parietalis and capsularis taken from women treated with or without mifepristone.	80
Fig. 4.5. PRL production released by cultured term decidual cells treated with or without oestradiol and progesterone, either alone or in various combinations.	93
Fig. 5.1. Comparison of progesterone production (A) and PRL production (B) in decidua parietalis and decidua capsularis in vitro during a 5 day incubation period.	108
Fig. 6.1. The effect of PCM on the release of decidual PRL from term pregnancy decidua.	118
Fig. 6.2. The effect of PCM on the release of decidual PRL from early pregnancy decidua.	119
Fig. 6.3. The effect of hCG on the release of PRL from early and term pregnancy decidua.	121
Fig. 6.4. The effect of dbcAMP on the release of PRL from term decidual cells.	122

Fig. 6.5.	The effect of TPA on the release of PRL from the cultured decidual cells.	123
Fig. 6.6.	The effect of oestradiol, progesterone and TPA on decidual PRL production of term decidual cells.	124
Fig. 7.1.	Schematic representation of the predicted dopamine D2 receptor structure encoded by the clone pZ19.12 cDNA.	132
Fig. 7.2.	The amino acid and nucleotide sequence of the rat dopamine D2 receptor.	136
Fig. 7.3.	The amino acid and nucleotide sequence of the human dopamine D2 receptor sequence.	137
Fig. 7.4.	The effect of bromocriptine on decidual PRL production from untransfected term decidual cells during a 3 day period of in vitro culture.	140
Fig. 7.5.	The effect of oestradiol, progesterone and bromocriptine on decidual PRL production from term decidual cells during a 3 day in vitro culture observed in two patients.	141
Fig. 7.6.	The effect of bromocriptine on decidual PRL production of term decidual cells transfected with rat dopamine D2 receptor construct.	154
Plate 4.1	parietalis and capsularis from women treated without or with mifepristone in vivo.	51
Plate 4.2.	Immunocytochemical staining of PRL and in situ localisation of PRL mRNA in dispersed decidual cells from human term decidua.	59
Plate 5.1.	Progesterone and oestrogen receptor distribution in human early and term pregnancy decidua analysed by immunocytochemistry.	105
Plate 7.1.	in situ localisation of dopamine D2 receptor with immunocytochemistry in control and transfected human myometrial cells.	142
Plate 7.2.	Transcriptional analysis of dopamine D2 receptor mRNA in human early or term decidua by Northern blot analysis.	144
Plate 7.3.	Reverse transcription-PCR analysis of the dopamine	145

List of Plates

	Page
Plate 2.1. The development of an in situ hybridization method to localize decidual PRL mRNA using [³⁵ S]-hPRL cDNA probe in utero-placental unit.	33
Plate 2.2. The development of the immunocytochemistry method to localize decidual PRL in utero-placental unit.	43
Plate 2.3. Autoradiograph of a Northern blot showing the hybridization of [³² P]-hPRL cDNA with RNA from ovine pituitary and human term decidua.	49
Plate 2.4. Immunocytochemical staining of PRL and in situ localization of PRL mRNA in utero-placental tissues from early and term pregnancies.	50
Plate 3.1. In situ localization of PRL mRNA and immunocytochemical staining of PRL in human decidua from early, mid and term pregnancies.	59
Plate 3.2. Autoradiograph of a Northern blot showing the hybridization of [³² P]-hPRL cDNA with RNA from human early and term decidua.	62
Plate 4.1. Immunolocalization of PRL in decidua parietalis and capsularis from women treated without or with mifepristone in vivo.	81
Plate 4.2. Immunocytochemical staining of PRL and in situ localization of PRL mRNA in dispersed decidual cells from human term decidua.	89
Plate 5.1. Progesterone and oestrogen receptor distribution in human early and term pregnancy decidua analysed by immunocytochemistry.	105
Plate 7.1. In situ localization of dopamine D2 receptor mRNA in untransfected and transfected human decidual cells.	142
Plate 7.2. The autoradiographic detection of dopamine D2 receptor mRNA in ovine and human early or term decidua by Northern blot analysis.	145
Plate 7.3. Reverse transcriptase PCR analysis of the dopamine	146

The Background of This Thesis

1.1. Introduction

Prolactin (PRL) production is normally restricted to the anterior pituitary gland. In many mammals, however, PRL is also produced by the decidualized uterine endometrium late in the luteal phase of the menstrual cycle and on through pregnancy shortly before implantation occur (Riddick et al., 1978; Maslar and Riddick, 1979). Decidual PRL bears the striking homologies with the pituitary PRL with regards to its chemical, biological and immunologic properties. Although much less is presently known about the regulation and function of decidual PRL in contrast to the intensive effort directed to understanding of pituitary PRL, its regulation appears to be apparently different from PRL in the pituitary, to which the different regulatory system of gene structure and subcellular storage sites might contribute. Decidual PRL probably serves different functions related to the fetus, maternal and placenta.

This review will deal with, first, the anatomical and histological structure of human endometrium, the process of decidualization and related factors, and then the tissue and cellular origin of decidual PRL, the regulatory mechanism and relevant molecular basis, and its function in the utero-feto-placental unit.

1.2. Human endometrium and decidualization

1.2.1. The cellular structure of human endometrium

There are two layers of endometrium in the uterus, i.e. the innermost layer is the functional layer and the outer layer is the basal layer (Pincus et al., 1975). The functional layer consists of the uterine epithelium and the underlying stroma. The uterine epithelium is a simple cuboidal epithelium which forms a continuous layer on the surface of the uterus. The glands grow further down into the stroma, their density depending on the

CHAPTER 1

The Background of This Thesis

1.1. Introduction

Prolactin (PRL) production is generally restricted to the anterior pituitary gland. Uniquely in human and primates, however, PRL is also produced by the decidualized uterine endometrium late in the luteal phase of the menstrual cycle and on through pregnancy should blastocyst implantation occur (Riddick et al., 1978; Maslar and Riddick, 1979). Decidual PRL bears the striking homologies with the pituitary PRL with regards to its chemical, biological and immunologic properties. Although much less is presently known about the regulation and function of decidual PRL in contrast to the intensive effort directed to understanding of pituitary PRL, its regulation appears to be apparently different from PRL in the pituitary, to which the differently regulatory region of gene structure and subcellular storage sites might contribute. Decidual PRL probably serves different functions related to the fetus, amniotic fluid homeostasis and placenta.

This review will deal with, first, the anatomical and histological structure of human endometrium, the process of decidualization and related factors, and then the tissue and cellular origin of decidual PRL, the regulatory mechanism and relevant molecular basis, and its function in the utero-feto-placental unit.

1.2. Human endometrium and decidualization

1.2.1. The cellular elements in human endometrium

There are four main tissues situated in the human endometrium, i.e. the luminal epithelium, glandular epithelium, stroma and vascular vessels (Findlay et al., 1990). The columnar epithelial cells on the uterine luminal surface, along with the epithelial cells of the glands, form a continuum over the luminal surface of the uterus. The glands grow further down into the stroma, their density depending on the

hormonal status. The extracellular matrix of the endometrium is composed of collagen fibres, in which are embedded smaller fibrocyte-like stromal cells which will undergo the dramatically morphological and biochemical changes during normal menstrual cycle and pregnancy. In addition, plasma cells, macrophages and lymphocytes have also been identified within the stroma. The blood vessels in the stroma area are mainly formed by smooth muscle cells lined with endothelial cells.

The decidua is the endometrium of the uterus of pregnancy and is so named because much of it is shed after parturition. During pregnancy, the decidua thickens; eventually, a depth of 5 to 10 mm is attained. The portion of the decidua directly beneath the site of implantation forms the decidua basalis; that portion that overlies the developing ovum and separates it from the rest of the uterine cavity is the decidua capsularis. The remainder of the uterus is lined by decidua parietalis. During the early months of pregnancy, there is a space between the decidua capsularis and the decidua parietalis since the gestational sac does not fill the entire uterine cavity. By the fourth month, the enlarging sac fills the uterine cavity; and with fusion of the decidua capsularis and parietalis, the uterine cavity is obliterated. The decidua capsularis is most prominent at about the second month of pregnancy; at this time, it consists of decidual cells that are covered by a single layer of flattened epithelial cells without traces of glands; internally, it is in contact with the chorion laeve (Pritchard et al., 1985).

1.2.2. The subcellular sites of action of progesterone and oestrogen

The uterine cells undergo intense periods of proliferation, differentiation and then secretory activity during the normal menstrual cycle and pregnancy, initiated and modified by hormones, among which oestrogen and progesterone play specific roles.

Oestrogen and progesterone are produced by the corpus luteum during the maternal menstrual cycle and corpus luteum/placenta during pregnancy. There is a general agreement that the sex steroids enter into cells by passive diffusion because of their lipophilic properties,

although it is difficult to rule out a facilitated transport of steroids into target cells through specific carrier molecules (Sakamoto et al., 1986). However, different suggestions regarding the mechanism of action of sex steroids within the cells have been put forward.

The classic model was described as: steroid hormones interacted with cytosolic binding proteins. The hormone-receptor complex undergoes a process termed "activation" that resulted in an enhanced affinity of steroids for chromatin, followed by initiation of new nucleic acid and protein synthesis (Gorski and Gannon, 1976; Sakamoto et al., 1986). This classic theory was mainly based on the results from subcellular fraction studies.

The study of steroid receptor biochemistry started with the pioneering works of Jensen and Jacobson (1962) with oestradiol. Soon identical studies were performed for both oestrogen and progesterone receptors by employing steroid binding assays (Bayard et al., 1978; Levy et al., 1980; Padayachi et al., 1987). All of the studies of oestrogen and progesterone receptors in the endometrium have analysed both cytosolic and nuclear receptors with high and specific affinity for their ligands (West and Brenner, 1985).

It has been suggested that the ratio of cytoplasmic and nuclear receptors was under the influence of their ligands. When the circulating level of sex steroids increased, there was a rise of the respective receptors, but mainly in the nuclear compartment, as in the case of normal menstrual cycle (Bayard et al., 1978). In addition, the acute injection of oestrogen and progesterone resulted in the increase of the steroid-binding components in the nuclear fraction (King et al., 1965; Gorski et al., 1968; O'Malley et al., 1971; Mueller et al., 1972; Jensen and DeSombre, 1973; Feil and Bardin, 1975; Gorodeski et al., 1987). These results led to the conclusion that within the cell oestrogen and progesterone interacted with the unoccupied steroid hormone receptors forming a hormone-receptor complex upon hormone exposure. This process induced steroid receptor transformation to the actively biochemical form that was capable of penetrating the nucleus and binding to the specific region of chromatin, followed by a series

responses within the target cells (Gorski et al., 1968; Jensen and DeSombre, 1972).

However, this classical view about the mechanism of steroid action has been challenged within the past few years. Initially using cellular enucleation method without the dilution/extraction artifacts, the receptor-DNA ratio was constant in all fractions, suggesting that unoccupied oestradiol receptors were loosely bound to the nucleus and only released into the cytosol during the extraction procedure (Welshons et al., 1984). Then, with the development of highly specific monoclonal antibodies of steroid receptors which were able to recognize both unoccupied and occupied steroid receptors (Press and Greene, 1988), it became possible to visualize the receptor proteins in situ in the intact cells. Most of the immunocytochemical procedures were performed on the frozen sections to avoid any procedures which could result in redistribution and extraction of steroid receptors (King and Greene, 1984; Press and Greene, 1984; Bergeron et al., 1988b; Garcia et al., 1988). The results from the immunocytochemistry studies revealed that the specific staining of steroid receptors was only localized in the nucleus and no detectable cytoplasmic staining at the level of the light microscope has been reported yet. Furthermore, it has been demonstrated that the newly synthesized progesterone receptors in the cytoplasm in the absence of ligand rapidly entered the nucleus (Guiochon-Mantel et al., 1989). However, with electron microscopy, there were small amounts of progesterone receptor localized in the cytoplasm (Perrot-Appianat et al., 1986).

Taken collectively, a new view of the mechanism of the steroid action has been proposed: steroid receptors were nuclear proteins which were synthesized in the cytoplasm in absence or presence of steroid hormones and rapidly entered the nucleus where they became loosely bound to the chromatin. Only occupied and transformed receptors were tightly bound to chromatin to induce the responses in the target cells. Their appearance in the cytosol in the subcellular fraction studies presumably reflected the fact that some nucleoplasmic proteins were released from the nuclei after tissue disruption during homogenization (Gorski et al., 1986; Sakamoto et al., 1986; Savouret et al., 1988; Brenner et al., 1990).

1.2.3. The regulation of oestrogen and progesterone receptors

Studies on the regulation of oestrogen and progesterone receptor contents in target tissue are fundamental to the understanding of steroid hormone action. Despite the drawback of the ligand binding assays as discussed above, they remain the standard quantitative methods for analysing steroid receptors.

Progesterone receptor was controlled by oestradiol and its own ligands. This general model of double regulation (oestrogen and progesterone) of progesterone receptor has been confirmed in the studies on the endometrium of higher non-human primates and the human. In the follicular phase of normal menstrual cycle, the level of progesterone receptor was parallel to the oestradiol level in the serum, whereas during the luteal phase there was a decrease in progesterone receptor as the progesterone produced by corpus luteum increased (Bayard et al., 1978; Kreitmann-Gimbal et al., 1980). Upon oestrogen administration, it has been further revealed that progesterone receptor was oestradiol induced and dependent (Gorodeski et al., 1988). However, this oestrogen induced increasing progesterone receptor was prevented by injection of progesterone in the artificially induced endometrial cycle in the women with gonadal dysgenesis or postmenopausal women (Kreitmann-Gimbal, et al., 1979; Lubbert et al., 1982). These observations reinforced results from an in vitro study where physiological concentration of oestrogen promoted the dose-dependent increases in progesterone receptor level in human endometrial cells (Eckert and Katzenellenbogen, 1981).

In terms of the regulation of oestrogen receptor, the increase of oestrogen level in the plasma in normal menstrual cycle (Kreitmann-Gimbal et al., 1980; lubbert et al., 1982; Ghosh and Sengupta, 1988) and in the artificially induced cycle (Tseng and Gurpide, 1975; West and Brenner, 1985) resulted in an increase of oestrogen receptor, while progesterone appeared to down-regulate oestrogen receptor. Thus these results suggested the concept that oestrogen receptor was stimulated by its own ligands and inhibited by progesterone.

The steroid binding assay allows quantification of the receptor, but it can not identify variations of steroid receptors in different cell types. Nevertheless, the detailed investigation of the cellular change in both oestrogen and progesterone receptors using immunocytochemistry have confirmed the general regulation model of sex steroid receptors obtained from steroid binding assays, that is oestrogen upregulated both sex steroid receptors, while progesterone caused down-regulation of both oestrogen and progesterone receptors (West et al., 1987; Bergeron et al., 1988a; Bergeron et al., 1988b). However, immunocytochemistry also showed that variations of steroid receptors existed in different cell types during the different stages of the menstrual cycle. Oestrogen receptor content changed synchronously in both glandular epithelia and stromal cells upon the different hormonal status of the menstrual cycle (Welshons et al., 1984; Lessey et al., 1988). The distribution of progesterone receptor in the endometrium seemed to be cell type dependent and had complex patterns. The depletion of progesterone receptor was most marked in glandular epithelium when progesterone levels rose during luteal phase, while stromal cells still maintained a significant progesterone receptor content (Lessey et al., 1988; Press et al., 1988). In ovariectomized monkeys progesterone receptor was restricted to the glandular epithelium, while, after oestrogen treatment, progesterone receptor was observed in luminal and glandular epithelia as well as stromal cells and oestrogen enhanced the staining intensity of oestrogen receptor in all cell types (Okulicz et al., 1989). Segregation of function through alteration of receptor content may be an important mechanism in steroid dependent growth and differentiation of target tissue.

The presence of oestrogen and progesterone receptors was also observed by immunocytochemistry in the muscle cells of uterine arteries, but not in the endothelium of uterine arteries nor in uterine capillaries or veins (Perrot-Applanat et al., 1988). The intensity of progesterone receptor staining in the uterine vasculature was still under the control of oestrogen. These results suggested that sex steroid hormones may

regulate uterine blood flow through a direct effect on uterine arterial walls.

Recently, a new concept of stromal-epithelial interaction has been put forward which was based on the study of the luteal-follicular transition period. In either natural or artificial luteal-follicular transition periods, oestrogen and progesterone receptors were only detectable in stromal but not epithelial cells, when the epithelial cells underwent various oestrogen and progesterone-dependent events (Brenner et al., 1990). These results suggested the presence of soluble mediators that transmitted information between the stroma and epithelium in the endometrium.

Although the regulation of steroid receptors of non-pregnant uterus has been extensively examined, there is little information about the control of sex steroid receptors during pregnancy. Oestrogen receptors in decidua could be increased by treatment with RU 486 implying that the inhibitory action of progesterone on oestrogen receptors still existed in the decidua during pregnancy (Haluska et al., 1990). This was further confirmed by in vitro decidual cell culture (Takeda and Leavitt, 1986). The results from binding assays showed that the decidua in early pregnancy was characterized by a large concentration of progesterone receptor (Levy et al., 1980) and both sex steroid receptors could be identified in term decidua (Khan-Dawood and Dawood, 1984). The latter study was disputed by the results from Padayachi (Padayachi et al., 1987) in which oestrogen and progesterone receptors decreased to undetectable level at term pregnancy. During pregnancy the local factors derived from placenta might play an important role in the regulation of steroid receptors.

1.2.4. Decidualization

1.2.4.1. Morphological and biochemical changes in stromal cells associated with decidualization

Decidualization begins in the human endometrium during the last week of normal ovulatory cycle, even in the absence of conception

(Maslar and Riddick, 1979). The transformation of stromal cell into decidual cell was characterized by the increased cell proliferation, morphological changes and an alteration with biochemical features of all cells.

In the of follicular phase of menstrual cycle, before decidualization, there is an active proliferation of stromal cells in the endometrium. These stromal cells then differentiate in the late luteal phase and pregnancy into decidualized cells. The stromal cells enlarged when they started to decidualize and continued to increase in size as they gradually differentiated into the large epithelium-like polyhedral decidual cells (DeFeo, 1967). Decidualization first appeared in the vicinity of the spiral arterioles at day 22-23 of the menstrual cycle. By day 25, the decidual reaction has spread from the periarteriolar region into the subcapsular region, and during the final 2 days of the cycle the upper two-thirds of endometrium are composed of the decidualized cells (Finn, 1977). If pregnancy occurred, the decidual reaction persisted. The ultrastructural changes induced by decidualization in the stromal cells consisted of an increase in rough endoplasmic reticulum, filaments (Enders et al., 1985) and development of gap junctions which were considered as ultrastructural marker of decidual differentiation (Lawn et al., 1971).

Decidual cells rich in glycogen and lipid (Glasser, 1986) were surrounded by basement membrane components that contained laminin, fibronectin, type IV collagen and heparan sulfate proteoglycan (Wewer et al., 1985). Of these substances, laminin and fibronectin were derived from decidua tissue (Irwin et al., 1989). The changes associated with enzymatic activities, such as alkaline phosphatase, sulfatase and cell proliferation, have been used as markers of decidualization (Bell, 1983; Irwin et al., 1989; Benedetto et al., 1990).

1.2.4.2. The control of decidualization

It is widely accepted that in the human endometrium the induction of decidualization is solely under the influence of ovarian steroid hormones. Extensive histological studies have confirmed that during

natural ovulatory cycle or artificially induced cycle with sequential treatment of oestrogen and oestrogen with progesterone, decidualization occurred in the late luteal phase (Dallenbach-Hellweg, 1988). Moreover, during ectopic pregnancy stromal cells also underwent decidual reaction. This in vivo model emphasized that decidualization in human, in contrast to animals, was a spontaneous phenomena that occurred in the presence of functional corpus luteum and did not require the local presence of an embryo. Studies in vitro confirmed that decidualization was induced by treatment of proliferative endometrium with progesterone alone during in vitro culture, and maintenance of decidualization was dependent on progesterone (Daly et al., 1983a; Maslar and Ansbacher, 1986).

While the role of oestrogen on decidualization is less defined, Daly et al (1983a) reported that oestrogen slowed the rate of progression of progesterone induced decidualization assessed histologically. In contrast, Irwin et al (1989) observed that concurrent oestrogen treatment could enhance progesterone induced-decidualization upon cell culture, which was consistent with the known induction of progesterone receptor by oestrogen in human endometrial cells as discussed before. Moreover, oestrogen was clearly the major stimulator of cell growth and proliferation in uterus (Pavlik and Katzenellenbogen, 1978), which was a direct prerequisite for decidualization (Bell, 1983). Furthermore, oestrogen priming was important for the subsequent protein secretion by decidua (Seppala et al., 1988). Thus, oestrogen might be indirectly involved in the control of decidualization.

During pregnancy, the factors from feto-placental unit may augment and maintain the decidualization initiated by steroid hormones. Firstly, large amounts of progesterone and oestrogen derived from placenta replace that produced by corpus luteum to maintain decidualization during pregnancy. Insulin-like growth factor (IGF) which is involved in proliferation of a number of cell types and its binding protein have been localized in decidual cells (Bell, 1989), implying potential role of IGF in regulating decidual cell differentiation.

In addition, a number of other proteins, such as pregnancy-associated endometrial $\alpha 2$ -globulin ($\alpha 2$ -PE α), and epidermal growth factor, have also been suggested as possible factors in the induction of decidualization (Waites, et al., 1988, Yamamoto et al., 1989).

1.2.4.3. The physiological and endocrine role of decidua

Decidual cells contain high concentrations of glycogen and lipid implying a potential role for supplying nutrition to the blastocyst (Kearns and Lala, 1983). In vitro studies of interaction between human trophoblast and endometrium, showed that the trophoblast invasion was under the control of decidua (Kishimoto et al., 1987). Decidual cells were also believed to play a role in the protection of the embryo from maternal immune rejection (Golander et al., 1984). After decidualization, decidual cells were of functional secretory activity, and a variety of proteins have been isolated from human endometrium (Table 1). The exact role of these proteins remains to be clarified but some of them, especially PRL, have generally been accepted as the markers of decidualization (Yamamoto et al., 1989; Irwin et al., 1989).

$\alpha 2$ pregnancy-associated endometrial globulin	Bell 1986
Pregnancy-associated plasma protein-A	Sjoberg et al., 1984
Placental protein 5	Butzow et al., 1986
Endometrial protein 1-17	Bell, 1986
Epidermal growth factor	Yamamoto, 1989
Estrene sulfate sulfate	Baradatta, 1990
Lipocortin-I	Shacke et al., 1991

Table 1.1. Human endometrial proteins

Proteins	References
Prolactin	Tyson et al., 1972
Placental protein 12	Whalstrom & Seppla, 1984
Endometrial protein 14	Bell, 1986
α -1 pregnancy-associated endometrial globulin	Bell, 1986
Insulin-like growth factor-binding protein	Koistinen et al., 1986
Placenta protein 14	Julkunen et al., 1986
Progesterone-dependent endometrial protein	Joshi et al., 1980
Endometrial protein 15	Bell, 1986
α -2 pregnancy-associated endometrial globulin	Bell, 1986
Pregnancy-associated plasma protein-A	Sjoberg et al., 1984
Placental protein 5	Butzow et al., 1986
Endometrial protein 1-17	Bell, 1986
Epidermal growth factor	Yomamoto, 1989
Estrone sulfate sulfatase	Benedetto, 1990
Lipocortin-I	Pihoker et al., 1991

1.3. Identification of Decidual Prolactin

In the early 1970's, high levels of PRL were found in amniotic fluid throughout gestation (Tyson et al., 1972). Since then, extensive studies have been carried out to identify the similarities and dissimilarities between pituitary PRL and PRL derived from amniotic fluid. On the basis of molecular size, net charge and isoelectric point upon polycrylamide gel electrophoresis and isoelectric focusing, human PRL from amniotic fluid is indistinguishable from pituitary PRL (Ben-David et al., 1973). Again this PRL was identical to pituitary PRL according to its chromatographic (Frame et al., 1979a), immunologic, biologic (Tomita et al., 1982a; 1982b), and receptor binding properties (Golander et al., 1978). In addition, cDNA for decidual PRL has been prepared and the amino acid sequence predicted from the nucleotide sequence of its cDNA was identical to pituitary PRL (Takahashi et al., 1984).

1.4. The source of decidual PRL

Initially the major source of PRL in amniotic fluid was thought to be from either maternal pituitary since the concentrations of PRL in maternal plasma was positively correlated with amniotic fluid PRL concentration and radiolabelled PRL could pass from the mother to the amniotic cavity (Josimovich et al., 1974), or fetal origin since the molecular size heterogeneity of PRL in amniotic fluid differed from maternal PRL to a greater degree than fetal PRL (Fang and Kim, 1975). An extra-pituitary origin of amniotic fluid PRL was suggested as result of experiments in monkeys in which neither maternal hypophysectomy nor fetal death decreased the concentration of PRL in amniotic fluid (Walsh et al., 1977). This indicated that neither the maternal pituitary gland nor a live fetus was necessary for the PRL accumulation in amniotic fluid. An extra-pituitary source of amniotic fluid PRL in women was further suggested since suppression of maternal and fetal pituitary PRL to very low levels with bromocriptine throughout pregnancy was not associated with any decline in the level of amniotic fluid PRL (Bigazzi et al., 1979a). Furthermore the changes in amniotic fluid PRL were not closely related in many cases with the changes in

plasma concentrations of PRL in either the mother or the fetus (Rosenberg et al., 1980).

The precise tissue origin of amniotic fluid PRL remained to be defined until Riddick and Kusmik (1977) reported that among the tissues of the amniotic membrane, i.e. the chorion, placenta and decidua, the amount of PRL contained solely in decidua was significant enough to explain the origin of PRL in amniotic fluid. Moreover, neither the initial PRL content in the decidua nor the amount of PRL accumulated in the medium during in vitro tissue culture was significantly different when the tissues from normal and ectopic pregnancies were compared (Maslar et al., 1980). Therefore, it was suggested that the presence of cells of fetal origin within uterus was not essential for the production of PRL by the decidua of early pregnancy. Amniotic fluid PRL levels in early pregnancy were much higher than those of maternal serum in normal pregnancy (Schenker et al., 1975). However amniotic fluid PRL levels obtained from the ectopic pregnancy were found to be essentially within the normal serum range (Rosenberg, 1984), providing further support for an essential role of decidual tissue as the source of amniotic fluid PRL in vivo. PRL was then shown to be synthesized and secreted in the endometrium during the normal menstrual cycle (Maslar and Riddick, 1979) and during cycles artificially induced by sequential administration of sex steroid hormones (Taga et al., 1982), confirming that the production of PRL by the endometrium did not depend upon either conception or implantation. This concept was confirmed by experiments in vitro in which endometrium from the proliferative phase of the cycle was treated with progesterone and underwent decidualization and then secreted PRL. In this system, the initiation of PRL secretion in vitro corresponded to the onset on histologically apparent decidualization (Daly et al., 1983a; Maslar et al., 1986) and the degree of decidualization of stromal cells was proportional to the increase of PRL produced by endometrium (Morimoto, 1988).

Confirmation was achieved by identification of a specific mRNA for decidual PRL by Northern Blot analysis using [³²P]-labelled cDNA probes (Clements et al., 1983) and an in vitro translation system (Taii et al., 1984). Only decidua contained PRL mRNA: no PRL mRNA was

found in amnion, chorion or trophoblast confirming that the PRL gene was indeed expressed in human decidua.

While these studies suggested that decidua in the utero-feto-placental unit was the only source of PRL in amniotic fluid, PRL had also been localized by immunocytochemistry in the amnion (Healy et al., 1977), chorion (Fram et al., 1979b; Bryant-Greenwood et al., 1987), the trophoblast (Al-Timimi and Fox, 1986) and in the cytoplasm of decidual cell (Meuris et al., 1980; Pileri et al., 1982; Kauma and Shapiro, 1986). As immunocytochemistry can not distinguish the sites of the synthesis from the sites of accumulation, it was not clear whether PRL was synthesized in all the PRL immunopositive cells or whether it is sequestered from an extracellular source.

The exact cell type which is responsible for PRL production in decidua has not been conclusively localized. Several groups have attempted to isolate the PRL producing cells by a number of different methods. DeZeigler and Gurpide (1982) reported that PRL producing cells could be selected by taking advantage of slowness of their attachment to the culture dishes. Similarly PRL producing cells could be obtained from term decidua employing isopycnic centrifugation (Markoff et al., 1983a). Later, Braverman et al. (1984) isolated purified decidual cells, distinguishable from epithelial cells, from early and mid pregnancy decidua which possessed immunocytochemically localized PRL and secreted PRL. According to different size of cells, PRL producing cells could be separated from fibroblast cells by selective filtration (Hochner-Celnikier et al., 1984). Results from these studies showed that PRL producing-cells were morphologically similar to decidual cells.

1.5 Biochemical fate of decidual PRL

The relatively low tissue contents of PRL measured in decidua (Golander et al., 1978) and in isolated decidual cells (Markoff, 1983a) and the finding of inhibition of PRL synthesis by cycloheximide treatment in explants of decidua (Riddick et al., 1978) suggested that there was only limited storage of PRL in decidual cells. Further evidences were obtained by the studies that different subcellular storage

sites existed in decidua- and pituitary-derived PRL following differential and density gradient centrifugations (Handwerger et al., 1984). Almost all of the PRL in the pituitary was contained in mitochondrial and microsomal particulate fractions. Following centrifugation on sucrose density gradients, they sedimented in a fraction associated with secretory granules. In contrast, decidual PRL was detected in the post-microsomal supernatant, and was distributed diffusely throughout the gradients, suggesting that the PRL in decidua was not stored in secretory granules. Moreover, no chromogranin, a major protein component of secretory vesicles, was localized in the decidual cells by immunocytochemical techniques, in contrast again to the strongly positive staining in pituitary cells (Handwerger and Capel, 1985). It is therefore concluded that decidual PRL is not stored in the tissue and it is secreted soon after its synthesis.

After PRL is released from decidua, it will be accumulated in the amniotic fluid by passing through the amniotic membrane. This assumption has been confirmed by in vitro study employing a double chamber system divided by amniotic membrane. The large quantities of PRL produced by the decidua crossed the fetal membrane to the amniotic side readily (Riddick and Maslar, 1981). This study was extended to demonstrate that the newly synthesized human decidual PRL was transported from the maternal to the fetal surface of the membrane within 4 hours and the biologic activity of decidual PRL determined by bioassay was retained after its transport (McCoshen and Barc, 1985). However, such transport required the intimate adhesion of decidua to amniochorion (McCoshen et al., 1982a).

During the normal menstrual cycle, no PRL was detectable in the proliferative phase upon in vitro tissue culture. After day 22 to 24 days of the cycle, PRL could be measured in the medium of cultured decidua (Maslar and Riddick, 1979). Generally, most of endometrium dated 25 to 28 days contained measurable amounts of PRL. During very early pregnancy, a significantly higher concentration of decidual PRL accumulated in the cultured medium was associated with those tissues in which definitive villi were identified (>2 weeks post conception) compared to those in which the syncytiotrophoblast had not yet

differentiated extensively (<2 weeks post conception) (Maslar et al., 1980). The concentration of PRL in amniotic fluid appeared to peak between 16 to 20 weeks, and then decreased to a plateau around week 36 (Kletzky et al., 1985). This decrease was probably due to the dilution by the continually increasing volume of amniotic fluid, while PRL production by decidua was still constant (McNeilly et al., 1977).

1.6. The regulation of synthesis and release of decidual prolactin

1.6.1. Dopamine and thyrotropin-releasing hormone (TRH)

Although decidual PRL is indistinguishable from pituitary PRL, a diversity of studies have shown that factors regulating the synthesis and release of decidual and pituitary PRL were distinct. The first striking difference in control of PRL production was that dopamine and dopamine agonist drugs given in vivo (Bigazzi et al., 1979a) or in vitro (Golander et al., 1979) had no inhibitory effect on decidual PRL secretion. Similarly, Thyrotropin-releasing hormone, the potent stimulator of pituitary PRL, failed to affect PRL secretion from decidual in in vitro studies (Golander et al., 1979), the mechanism for which might be related to the lack of the corresponding receptors in decidual cells (Bigazzi et al., 1979a).

1.6.2. Oestrogen and progesterone

As the decidual cell is one of the main targets of oestrogen and progesterone, extensive studies have been carried out to examine the effect of these steroid hormones on decidual PRL production. Progesterone appeared to be essential for the initiation and maintenance of decidual PRL production in the endometrium from the normal menstrual cycle (Maslar and Ansbacher, 1986; Maslar et al., 1986; Maslar and Ansbacher, 1988). Decidual PRL production increased in response to progesterone in a dose and time dependent way (Casslen et al., 1990), with inhibition by the antiprogestosterone RU 486 (Bischof et al., 1986; Chen et al., 1989). The induction and maintenance of decidual PRL production was related to the mechanisms causing the decidualization (Maslar and Ansbacher, 1986) and the ability of

endometrium to produce PRL corresponded to the degree of decidualization (Maslar and Riddick, 1979).

The potential regulatory role of oestrogen is contradictory. Some studies showed that oestrogen alone had either no effect or decreased the rate of decidual PRL production (Daly et al., 1983a; Huang et al., 1987), while others demonstrated that oestrogen, especially in combination with progesterone, was able to stimulate or modify decidual PRL production (Randolph et al., 1990; Casslen et al., 1990). The modified effect of oestrogen on progesterone-induced decidual PRL production might be due to its action of increasing progesterone receptors in vivo and in vitro as stated before.

During pregnancy, the regulatory role of oestrogen and progesterone is less defined. Oestradiol alone was without effect on decidual PRL production (Daly et al., 1983b), while in combination with progesterone had the effect of maintaining PRL production observed in early pregnancy (Maslar et al., 1988). Progesterone alone seemed to have little or no effect on PRL production by decidua obtained from either early (Maslar et al., 1988; Ren and Braunstein, 1990) or term pregnancy (Rosenberg and Bhatnagar, 1984). However, in short-term cultured explants of decidua obtained at Caesarean section, progesterone was capable of stimulating decidual PRL secretion, which was abolished in labour-exposed decidua (Daly et al., 1983b). Thus the effect of progesterone may only be facilitative, maintaining decidual differentiation, and PRL production is only associated with the presence of progesterone-dependent differentiated decidual cells.

By examining decidual PRL mRNA levels in steroid hormone stimulated endometrium, it was shown that the increase in PRL production rate elicited by these sex steroids was accompanied by an increase of PRL mRNA in decidual cells (Huang et al., 1987). However, it is still not clear whether the accumulation of PRL mRNA in decidua was due to facilitated PRL gene transcription or PRL mRNA stabilization, or an overall increase in cellular metabolism, protein synthesis and cell differentiation.

1.6.3. Factors derived from the Utero-placental unit

It has been suggested that decidual PRL production is controlled by complex regulatory mechanism, operating at the local level. Insulin stimulated both acute secretion and de novo synthesis of decidual PRL (Thraikill et al., 1989). Insulin-like growth factor I (IGF-I) was also a potent stimulator, with marked effects on decidual PRL release, mediated through specific insulin-like growth factor I receptors (Thraikill et al., 1988). Human chorionic gonadotropin (hCG), a major protein produced by placenta, given repeatedly, failed to induce and stimulate PRL production from the endometrium of normal menstrual cycle (Ying et al., 1988), early pregnancy (Ren and Braunstein 1990) or term pregnancy (Handwerger et al., 1983). However, a single study reported a stimulating effect of hCG at a specific dose on decidual PRL secretion (Rosenberg and Bhatnagar, 1984).

A decidual PRL-releasing factor has been semi-purified from placenta and the conditioned medium from human placental explants (Handwerger et al., 1987a). This PRL releasing factor was heat (56°C for 30 minutes) and pH (pH 3.0-10.0) stable, unaffected by lipid extraction and sensitive to proteolytic digestion, with a molecular weight of 23.5 kilodalton . It was able to induce biphasic stimulation of synthesis and secretion of decidual PRL (Handwerger et al., 1983; Golander et al., 1988). In contrast, an inhibitory effect associated with a factor from decidua on decidual PRL secretion has been reported (Markoff et al., 1983b). Again, arachidonic acid, a precursor of prostaglandins, appeared to inhibit decidual PRL production in a dose dependent manner, although prostaglandins applied directly had no effect on PRL production by decidua (Handwerger et al., 1981), which suggested that arachidonic acid did not appear to be mediated by a cyclooxygenase product of arachidonic metabolism. Lipocortin-I, a family of calcium-dependent phospholipid-binding proteins present in the local environment derived from decidua and placenta inhibited decidual PRL production and blocked the stimulating effect of PRL production by decidual PRL-releasing factor (Pihoker et al., 1991). All these results demonstrated that local factors from utero-fetal-placental

unit played important roles in regulating decidual PRL production, although the relative importance of each of the factor remains unclear.

1.6.4. Intracellular signalling systems

A number of studies have investigated the relationship between decidual PRL production and the environment of the extracellular osmolality. In a longer term exposure of decidual explants to a hyperosmotic environment produced by sodium chloride within a range of 351 mmol/kg to 457 mmol/kg, decidual PRL production was significantly increased (Andersen et al., 1982). This effect was not specific to sodium chloride and also occurred at the specific hyperosmolality of 387 mmol/kg, with potassium chloride, mannitol, sucrose or choline chloride (Andersen et al., 1984). This hyperosmolality-affected PRL secretion from human decidua was prevented by ouabain, a cardiac glycoside blocking the Na^+/K^+ pump (Andersen et al., 1986). In addition, hyposmolality had an opposite, inhibitory effect on decidual PRL production (Andersen et al., 1982). All these results indicated that intracellular ionic concentrations were of importance in the regulation of the synthesis and secretion of decidual PRL in vitro. This concept has, however, been disputed by Markoff et al. (Markoff et al., 1982), who had shown no effect of changes in osmolality up to 336 mOsm/kg on decidual PRL production. Extracellular calcium was required for basal secretion of decidual PRL, although the calcium-calmodulin complex inhibitor trifluoperazine had no inhibitory effect on decidual PRL secretion (Richards et al., 1982).

Activation of the adenylate cyclase-cyclic AMP system and protein kinase C by 12-O-tetradecanoylphorbol-13-acetate (TPA), testing diacylglycerol/inositol triphosphate system, inhibited acute release of PRL production from decidua (Handwerger et al., 1987b; Harman et al., 1986), but it is still uncertain of the long term action of TPA on decidual PRL production. Gellersen et al. (1989b) reported that, in long term, TPA reduced PRL mRNA in the human B-lymphoblastoid cell line IM-9-P3, which ectopically produced PRL resembling decidual PRL with regard to its similar molecular size of PRL mRNA and identical 5' untranslated gene structure (DiMattia et al., 1990), very small

intracellular PRL pool and the unresponsiveness of those cells to many secretagogues controlling pituitary PRL secretion. These observation that both cAMP and protein kinase C activation inhibited the release of decidual PRL clearly indicated that there were striking differences in the intracellular mechanisms involved in the synthesis and release of decidual PRL from those of most other peptide hormones, including pituitary PRL.

Unlike pituitary PRL, decidual PRL was not stored in the secretory granules as discussed above, explaining perhaps the different mechanisms of control of PRL synthesis and secretion operation in these tissues. In addition, the different gene structure in the DNA regulatory region as discussed later in this review might be the main reason that resulted in the marked regulatory difference between decidua and pituitary PRL. Furthermore, decidual cells did not possess the cell surface gangliosides A2B5 and 3G5, which were features shared by all anterior pituitary and other endocrine cells that synthesize and release protein hormones (Handwerger and Capel, 1985). The decidual cells thus appear to represent a unique protein hormone-producing cells different from the classical protein hormone-secreting cells.

1.7. The physiological function of decidual PRL

1.7.1. The localization of PRL receptors in utero-feto-placental unit

Since interaction with receptors is assumed to be the first step in the mechanism of action of protein hormones, a mandatory prerequisite for any biological action of decidual PRL would be the demonstration of PRL receptor. PRL receptors have been identified in human chorion-decidua (McWey et al., 1982), chorion laeve (Herington et al., 1980) and amnion (McCoshen et al., 1982b; Jayatilak and Gibori, 1986). The binding parameters of PRL receptor in these tissues were similar to those of other recognised PRL target tissues. Similarly, immunocytochemistry techniques have revealed PRL in amnion (Healy et al., 1977), chorionic cytotrophoblast (Bryant-Greenwood et al., 1987)

1987) and placenta (Pileri et al., 1982). These studies suggested an auto/paracrine role of decidual PRL.

1.7.2. Effect of PRL on water and minerals across the fetal membrane

It has been suggested that decidual PRL might function as an osmoregulator, modulating water and electrolyte transport across the fetal membrane in a manner similar to the effects of PRL on water and ion transport in lower vertebrates. Several investigators have displayed a role for decidual PRL in the regulation of amniotic fluid volume and mineral content (Tyson et al., 1984; Raabe and McCoshen, 1986), as PRL altered the permeability of human chorioamnion to these materials (Holt and Perks, 1975; Leontic and Tyson, 1977; Stray-Pedersen, 1982). These findings were supported by an in vivo study in which the injection of PRL into the amniotic fluid of rhesus monkeys reduced and prevented the rapid changes in the movement of sodium, potassium and water across the fetal membrane following the alteration in tonicity of the amniotic fluid (Josimovich et al., 1977). Regardless of the experimental design, it appeared that the fetal membrane response was specific to the presence of PRL, since addition of anti-prolactin or anti-prolactin receptor antibodies completely negated the effect of PRL (Leontic and Tyson, 1977). Furthermore, a clinical study in the women with chronic polyhydramnios has shown there were a reduced number of PRL receptors in the chorion laeve of these patients (Healy, 1982; 1985). It was suggested that this chronic PRL receptor defect might cause the development of chronic polyhydramnios. Hence, there seems little doubt that PRL affects the water and minerals transport across the amniotic membrane and in human it may also protect the extracellular fluid volume of the fetus. However, decidual PRL only increased water flow from the fetal to the maternal compartment and when PRL was added to the maternal side of the amniotic membrane, no effect on water volume rate was noted (Andersen, 1982). Thus both the mechanism and the physiological role and importance of these effect of PRL on fluid balance remain to be established conclusively.

1.7.3. Effect on fetal lung maturation

Decidual PRL has also been implicated in the regulation of fetal lung maturation, through activation of surfactant synthesis in the basis of both experimental and clinical evidences (Hamosh and Hamosh, 1977; Grosso et al., 1980). PRL receptors appeared to exist in the fetal lung and PRL stimulated phosphatidycholine generation in explant cultures of fetal lung (Mendelson et al., 1981). Large quantities of decidual PRL are present in the amniotic fluid allowing decidual PRL gain access to the fetal tracheobronchial tree. However, such a role for decidual PRL on fetal lung maturation was disputed by a study showing an absence of a positive relationship between amniotic fluid PRL level and other indices of fetal lung maturity, such as, the ratio of lecithin/sphingomyelin and the palmitic/stearic acid (Diani and Perdelli, 1981).

1.7.4. Other effects of decidual PRL

In women, periarteriolar decidualization began from day 23 of the menstrual cycle and was exclusively under the control of oestrogen and progesterone. PRL was detected in endometrium after day 23 of the cycle and its production increased as decidualization spread through the endometrial stroma (Maslar and Riddick, 1979). Obviously this process proceeded precisely at the time when implantation normally occurs. Moreover, decidual PRL generation was low in infertile women with a luteal phase defect endometrium (Daly et al., 1981). These findings suggested a role for endometrial PRL in implantation. Furthermore, PRL receptors were apparently present in the decidual cells as discussed above, producing the basis for PRL interaction with decidual cells.

It has also been suggested that decidual PRL may be involved in the control of parturition through the mechanism of the modulation of prostaglandin synthesis in the fetal membrane (Healy, 1984; Tyson et al., 1985), the stimulation of oestrogen sulfatase activity of decidual cells to increase the level of oestrogen (Braverman and Gurpide, 1986), the regulation of the uterine contractility (Horrobin, 1973) and prevention of

the immunological rejection of the blastocyst and fetus (Karmali et al., 1974). However, conclusive support for many of these proposed roles remains to be investigated.

1.8. Decidual PRL gene expression

Under normal physiological conditions expression of the human PRL gene is tissue specific, restricted to the anterior pituitary and the decidua. Comparing the understanding of the regulatory mechanism of controlling PRL gene expression in pituitary with decidua, much less is known about the regulation of the gene expression in decidua.

Decidual PRL was first localized in the term decidua-chorion by Northern blot hybridization, with a demonstration of a similar sized PRL mRNA coding for PRL in decidua and pituitary when comparing PRL mRNA from human decidua-chorion with PRL mRNA from ovine pituitary (Clements et al., 1983). The existence of PRL gene expression in decidua was further confirmed by an in vitro translation system, in which only decidua contained detectable amounts of PRL mRNA among amnion, chorion and decidua (Taii et al., 1984).

Further work was carried out to determine the similarities and dissimilarities between the PRL gene sequence in the pituitary and decidua in order to understand the regulation of PRL gene expression in decidua. The nucleotide sequence of decidual PRL mRNA was determined from a 807 base pair cDNA clone that contained a coding region corresponding to 18 amino acid residues of the signal peptide, 199 amino acids of the mature protein, and 157 nucleotides of the 3'untranslated region (Takahashi et al., 1984). This was very similar to the full structure of the pituitary PRL deduced from a 914 base pair cDNA clone of pituitary adenomas, consisting of a 28 amino acid signal peptide and 199 amino acids of the secreted protein (Cooke et al., 1981). It was therefore assumed that, since there was only a single copy of the human PRL gene, the human pituitary and decidual genes were identical in structure.

In contrast to the PRL protein coding sequence in decidua being identical to that in the pituitary, a recent study has revealed that decidual PRL mRNA was approximately 150 nucleotides longer than the transcript from the pituitary. The size difference to pituitary PRL mRNA persisted after RNase H digestion of Poly(A) tails, excluding the possibility the the elongation was due to a longer poly(A) tract (Gellersen et al., 1989a). The elongation was therefore not attributed to differences in the protein coding region or in the degree of polyadenylation and was suggested to reside in the 5' untranslated region of the molecule, which implied the usage of a transcription initiation site different from that in the pituitary PRL gene.

More direct evidence for the presence of elongated 5' untranslated regions of decidual PRL mRNA was obtained from the sequence of isolated decidual PRL cDNA. It has now been demonstrated that decidual PRL mRNA similar to the PRL mRNA from a human B-lymphoblast cell line IM-9-P3 and possessed identical 5' ends having 5'-untranslated sequence of 83-263 nucleotides longer than that of the pituitary PRL mRNA, located as a new 5'-noncoding exon 5-7 kilobase pairs upstream of the human pituitary PRL gene, exon 1 (DiMattia et al., 1990). A similar finding was obtained by Hiraoka et al. (1991), in which it was shown that the placental PRL mRNA was encoded by six exons including the placenta-specific exon, named as exon 0, which was located 5.3 kilobase upstream of exon 1 of the pituitary mRNA. In addition, the PRL gene in the decidua contained a placenta-specific promoter other than the pituitary-specific promoter, since the transcription of the PRL gene in decidua started at a site located 87 nucleotides or more upstream from the previously determined start site in pituitary.

The molecular mechanism underlying the pituitary-specific transcription of the PRL gene is the presence of a DNA-binding transcription factor (*trans*-acting factor), Pit-1/GHF1. This interacts with *cis*-acting elements located in the pituitary PRL gene regulatory region which was divided into the proximal and distal parts, each of which contained four binding sites for Pit-1 to direct transcription from PRL promoter in rat pituitary. In human pituitary, at least three

nuclear protein binding sites located in hPRL gene appear to bind with pit-1 (Davis, 1990). However, no Pit-1 binding consensus sequence was found within the 250 base pair sequence upstream of exon 0 (Hiraoka et al. 1991). Furthermore, no Pit-1 mRNA was expressed in decidua as revealed by polymerase chain reaction (PCR) (DiMattia et al., 1990) and Northern blot analysis (J. Brooks, J.R.G., Davis and A. McNeilly, unpublished observation, 1991). Thus, the mechanism of the different regulators controlling the synthesis and secretion of PRL from pituitary and decidua probably resides in the different regulatory structure of the PRL gene. Because *cis*-acting elements responsible for transducing regulatory signals from the cell membrane to the pituitary PRL gene are located in the immediate vicinity of the transcription initiation site associated with Pit-1 response elements (Elsholtz et al., 1986; Jackson and Bancroft, 1988), but this is not apparently the case for decidual PRL.

1.9. The aims of this thesis

The studies in this thesis were to investigate, in detail, the control of decidual PRL production by human decidua during pregnancy. Firstly, in Chapter 2, the exact cellular origin of decidual PRL was localized by co-localization of PRL and its mRNA in the utero-placental unit, as this was essential for the further determination of the relevant regulators of decidual PRL synthesis and release.

In Chapter 3, the relationship between decidualization and PRL production at the different stages of pregnancy was examined using *in situ* hybridization and Northern blot to assess PRL mRNA level, immunocytochemistry to examine the PRL content inside the decidual cells and radioimmunoassay to measure PRL in amniotic fluid. Further studies in Chapter 4 were carried out to reveal how important the effect of progesterone on decidual PRL production was during early pregnancy by blocking the action of progesterone with mifepristone (RU486) *in vivo*, and to examine the effect of both oestradiol and progesterone on PRL production by human term decidual cells. In Chapter 5, the potential cells involved in mediating the effects of oestradiol and progesterone on decidual PRL production were

determined by immunochemical localization of receptors for both steroids in human decidua.

In Chapter 6, the potential PRL releasing activity associated with the placenta and the intracellular signal transduction factors, cAMP and TPA, affecting PRL production, were investigated. Finally, in Chapter 7, the potential reasons for the failure of dopamine to affect decidual PRL secretion were examined by, firstly, determining whether dopamine receptors were expressed in decidual cells and, secondly, by determining whether introduction of the dopamine D2 receptors into decidual cells by transfection would induce responsiveness to dopamine.

In situ hybridization enables the precise localization and identification of individual cells that contain a specific nucleic acid sequence. When used together with immunocytochemistry, both mRNA transcribed from the gene itself and the gene product translated from mRNA can be identified. The aim of the study described in part I of this chapter was to develop an *in situ* hybridization technique using a cDNA probe to human pituitary PRL, for precise anatomical localization of PRL mRNA within human extra-placental unit. Thus detection of both PRL and its mRNA in a particular cell type, which gave strong evidence for the site of synthesis, would be obtained by combining *in situ* hybridization with immunocytochemistry.

To optimize *in situ* hybridization methodology with respect to morphology, sensitivity, and retention of target RNA, the following procedures were taken into account during developing this method:

1. Fixation;
2. Pretreatment of the tissue with;
3. Composition of hybridization buffer;
4. Temperature and duration of the hybridization;
5. The degree of washing after hybridization.

CHAPTER 2

2.1.2.1. Tissue collection and fixation

Localization of the Sites of Synthesis and Action of Prolactin by Immunocytochemistry and In Situ Hybridization within the Human Utero-placental Unit

Subcommittee of the Leishman Health Board, and all patients gave informed consent.

Part I. Development of an in situ hybridization method to localize PRL mRNA in utero-placental unit

2.1.1. Introduction

Tissue from term pregnancy was collected from patients with uncomplicated deliveries at 37-40 weeks. Fresh tissue In situ hybridization enables the precise localization and identification of individual cells that contain a specific nucleic acid sequence. When used together with immunocytochemistry, both mRNA transcribed from the gene itself and the gene product translated from mRNA can be identified. The aim of the study described in part I of this chapter was to develop an in situ hybridization technique, using a cDNA probe to human pituitary PRL, for precise anatomical localization of PRL mRNA within human utero-placental unit. Thus detection of both PRL and its mRNA in a particular cell type, which gave strong evidence for the site of synthesis, would be obtained by combining in situ hybridization with immunocytochemistry.

The fixed tissue was then processed on a standard tissue processor and To optimize in situ hybridization methodology with respect to morphology, sensitivity, and retention of target RNA, the following procedures were taken into account during developing this method:

1. Fixation;
2. Pretreatment of the tissue section;
3. Composition of hybridization buffer;
4. Temperature and duration of the hybridization;
5. The degree of washing after hybridization.

2.1.2. Materials and methods

2.1.2.1. Tissue collection and fixation

The studies in these thesis were approved by the Paediatric/Reproductive Medicine Ethics of Medical Research Subcommittee of the Lothian Health Board, and all patients gave informed consent.

Human early pregnancy tissue, calculated from the last menstrual period, was obtained by curettage during therapeutic abortions under normal anaesthesia. Tissue from term pregnancy was collected from patients with uncomplicated deliveries at 37-40 weeks. Fresh tissue was received at theatre/labour ward and put on ice immediately where early pregnancy tissue was isolated into decidua or trophoblast, while several small pieces of placenta and membrane including amnion, chorion and decidua were cut from placenta and fetal membrane. Tissue was then fixed in the following fixative:

1. 4% paraformaldehyde in 0.01 M Phosphate Buffer Saline (PBS), pH 7.4 for 24 h at room temperature (RT);
2. 0.25% and 2.5% glutaraldehyde in 0.01 M PBS, pH 7.4 for 24 h at 4°C.

The fixed tissue was then processed on a standard tissue processor and embedded in paraffin wax. The sections were cut at 5 μ M, floated onto poly-L-lysine coated slides (See **Appendix 1** for preparation of poly-L-lysine coated slides), and dried at 55°C overnight.

Frozen sections were assessed for comparing with the paraffin sections (See Chapter 5 for the method of preparation of frozen tissue). 5 μ M frozen sections were fixed in 4% paraformaldehyde for 10 min.

2.1.2.2. Pretreatment of the sections

For paraffin sections, the following schedules of pretreatment were tried (The method of preparation of all solutions used for in situ hybridization are detailed in **Appendix 2**):

1. Sections were pretreated with 0.2 M HCl for 20 min at RT and 2XSSC at 70°C for 10 min;
2. Pretreated with 0.2 M HCl for 20 min at RT and with prewarmed 2XSSC at 70°C for 10 min, followed by 1, 5 or 10 µg/ml proteinase K in 0.1M Tris/HCl, pH7.5, 2 mM CaCl₂ for 30 min at 37°C;
3. Pretreated with or without acetic anhydride.

2.1.2.3. Preparation of radiolabelled cDNA probe

The human PRL cDNA (0.96 Kb, ID p06715) was kindly donated by the Repository of Human DNA Probes and Libraries American Type Culture Collection, Rockville, MD, USA. It was labelled with [³⁵S] dCTP for in situ hybridization and with α[³²P] dCTP for Northern blotting by the method of Feinberg and Vogelstein (1983) using a random primed DNA labelling kit (Boehringer Mannheim, Lewes, Sussex, UK; the detailed labelling method is shown in **Appendix 3**).

2.1.2.4. Composition of hybridization buffer

By considering the factors affecting the stability of hybrids, such as salt strength, temperature, probe concentration and hybridization time, the following composition of hybridization solution was chosen (Preparation of stock hybridization solution is described in **Appendix 2**):

50%	deionised formamide
5X	SSPE
4%	dextran sulphate
0.1%	denhart's
200 µg/ml	tRNA
200 µg/ml	stDNA
0.1%	SDS
10 mM	DTT

Prehybridization buffer was prepared using DEPC treated water instead of dextran sulphate because it made the buffer too viscous and consequently was difficult to be removed prior to application of the hybridization buffer. The sections were incubated in 70 μ l of prehybridization buffer at 42°C for 1 h. In the meantime, the labelled probe was thawed and denatured in boiling water for 10 min, then transferred immediately to ice for 5 min to prevent reannealing of the probe, and added directly to prewarmed hybridization buffer. The buffer and probe were then maintained at 42°C in the oven until use.

2.1.3 Results

The hybridization was carried out at 42°C in hybridization buffer containing heat denatured [³⁵S]-PRL-DNA probe at a concentration of 15x10⁶ cpm/ml. Each slide containing 60-70 μ l hybridization buffer with probe was covered by an acetone cleaned coverslip, and kept in the humidified boxes. Different probe concentrations (1x10⁶ cpm/slide and 1.5x10⁶ cpm/slide) and hybridization time (18 h or 36 h) were tried to increase the signal-to-noise ratio.

Sections were treated with pancreatic RNase (20 μ g/ml) (Sigma) as a negative control in RNase buffer (20 mM Tris/HCl pH7.6, 1mM EDTA) for 30 min at room temperature before the hybridization procedure. The slides were then rinsed in 3 changes of 2XSSC (5 min each). The hybridization with probe was performed exactly as described above.

2.1.2.5. Washes

The following different washing steps were compared to assess the effect on background levels:

<u>Solutions</u>	<u>Schedule 1</u>	<u>schedule 2</u>	<u>Schedule 3</u>
2XSSC (RT)	4 X 30 min	2 X 1 h	1 X 2 h
1XSSC (RT)	4 X 30 min	2 X 1 h	1 X 2 h
0.5XSSC (RT)	1 X 1 h	2 X 30 min	1 X 1 h
0.5XSSC (37°C)	1 X 1 h	1 X 1 h	1 X 1 h

Following the washes, the slides were dehydrated by passing for three min each through an alcohol series (50%, 85%) containing 300 mM ammonium acetate to stabilize the hybrids, and then one change of 100% alcohol. After air drying, the slides were stored with cover at room temperature until ready to dip in emulsion. After coating with emulsion (Kodak), the slides were exposed at 4°C for 8 to 14 days, and then developed and stained with haematoxylin and eosin within 2 hours (See Appendix 4 for the detailed autoradiography).

2.1.3. Results

2.1.3.1. Fixation

In this study, two different cross-linking fixatives were compared (plate 2.1.). The decidua fixed in 2.5% glutaraldehyde (2.1.B) gave a reduced hybridization signal compared to that fixed in 4% paraformaldehyde (2.1.A). The tissue (decidua or trophoblast) was not fixed properly in 0.25% glutaraldehyde at 4°C for 24 h, so the tissue was not used for in situ hybridization.

2.1.3.2. Pretreatments

As shown in plate 2.1.C, without proteinase K pretreatment for paraffin sections in this study, no specific silver grains were observed over the sections. However, by comparing different concentrations of proteinase K (1, 5, and 10 µg/ml), the 5 µg/ml proteinase K treatment was the optimised concentration (2.1.A) because 1µg/ml proteinase K gave a lower signal, whereas no further increase in signal was observed on the sections when digested with 10 µg/ml proteinase K. For frozen sections, the hybridization signal was decreased dramatically by proteinase K treatment at all concentrations used above.

2.1.3.3. Probe concentration and duration of hybridization

After increasing the probe concentration from 1×10^6 cpm/slide to 1.5×10^6 cpm/slide, there was no further increment in signal (2.1.A and D). There was a prominent rise in signal after hybridization for 36 h

when compared with 18 h, although the background noise was also enhanced (2.1.E).

2.1.3.4. Washes

The best washing step developed in this study was the Schedule 3 which gave the best signal-to-noise ratio (2.1.A) compared with other two washing schedules. By using wash schedule 1 as shown in 2.1.F, there were no apparent grains observed over the section, with lower background noise indicating too high wash stringency used.

2.1.4. Discussion

The successful application of in situ hybridization depends on the quality of fixation of the tissue. Several aspects must be considered when preparing of tissue for in situ hybridization: target RNA retention, probe penetration, and preservation of morphology.

Two cross-linking fixatives, glutaraldehyde (0.25%, 2.5%) and 4% paraformaldehyde were used in this study. The results confirmed that glutaraldehyde fixation resulted in a lower hybridization signal even with proteinase K digestion than similarly treated paraformaldehyde fixed tissue. This apparently occurs because of a greater degree of crosslinking of proteins after fixation with glutaraldehyde than that with paraformaldehyde (Lawrence and Singer, 1985). It has been reported previously that at the same concentration, glutaraldehyde could preserve 20% more RNA than paraformaldehyde, but the accessibility for probes was decreased to 40% compared with paraformaldehyde (Singer et al., 1984). To compromise between RNA retention and probe penetration, 4% paraformaldehyde was therefore used as fixative for in situ hybridization throughout this study, since this gave sufficient morphology of tissue and better signal-to-noise ratio.

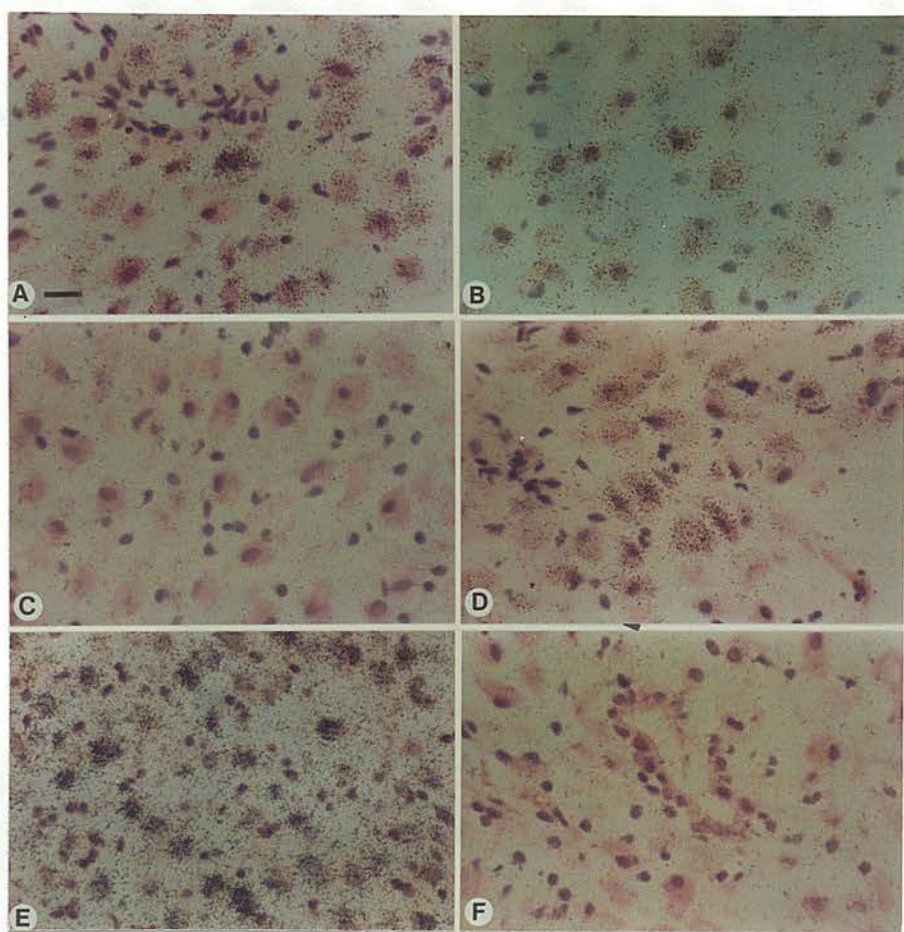


Plate 2.1.

The development of an in situ hybridization method to localize decidual PRL mRNA using [35 S]-hPRL cDNA probe in utero-placental unit. The scale bar is 20 μ m.

- (2.1.A.) In situ hybridization of PRL mRNA in human decidua fixed in 4% paraformaldehyde for 24 h with 5 μ g/ml proteinase K pretreatment. The probe concentration used was 1×10^6 cpm/slide and the hybridization was carried out at 42°C for 18 h followed by optimum posthybridization wash (Wash schedule 3).
- (2.1.B.) In situ hybridization of PRL mRNA in human decidua fixed in 2.5% glutaraldehyde for 24 h with identical pretreatment, hybridization and wash condition to 2.1.A. Note lower level of grains over the decidual cells than that shown in 2.1.A.
- (2.1.C.) In situ hybridization of PRL mRNA in human decidua under the same conditions as 2.1.A, but without treatment of proteinase K before applying the probe onto the sections. Note that grains were absent over the section.
- (2.1.D.) In situ hybridization of PRL mRNA in human decidua with increased amount of PRL cDNA probe (1.5×10^6 cpm/slide). Note no further increment of grains over the section compared with 2.1.A.
- (2.1.E.) In situ hybridization of PRL mRNA in human decidua with longer hybridization time (36 h). Note a prominent rise in the level of grains, but with enhancement of the background staining.
- (2.1.F.) In situ hybridization of PRL mRNA on the section of human decidua postwashed by schedule 1. Note grains were not apparent in the decidual cells indicating too high wash stringency used.

However proper tissue fixation varies with the physical property of the tissue, probe size, and the concentration of fixative used. This may explain why some investigators obtained better signal-to-noise ratio when glutaraldehyde fixative was used (Fox et al., 1985).

The aim of treatment of tissue after fixation, which is one of the more variable elements of in situ hybridization protocols, is to reduce non-specific binding and increase of probe penetration. The reduction of background in this study was attempted by inclusion of 10 mM DTT in the hybridization buffer to prevent the formation of disulphide bonds, prehybridization to block non-specific protein and nucleic acid interaction with probe, and acetylation with acetic anhydride to reduce non-specific electrostatic binding of the probe. Because acetylation was not effective in reducing the background in this study, this step was omitted in the routine procedure carried out in this study. This may be explained by the observation that the acetylation treatment may only reduce the background for the probe of more than 1500 bp, but not for smaller probes (Lawrence and Singer, 1985).

The need to facilitate probe diffusion depends on the type and extent of fixation, the particular tissue used, the length of probe, and section thickness. A permeabilization was required following paraformaldehyde fixation to allow access of the probe to mRNA in this study. This permeabilization included incubation of slides at high temperature (70°C), in diluted HCL (0.2 N), and with enzyme digestion (proteinase K). Increased signals were only obtained by combining these three treatments together. It appeared that enzyme digestion was an essential step of deproteinization because no specific signal was observed without application of proteinase k.

Since the routine paraffin sections were suitable for the retrospective study of PRL mRNA, and serial sections could be easily obtained for in situ hybridization and immunocytochemistry study, no further work was carried out on frozen sections.

The intention of the previous steps was to provide optimal conditions for the hybridization reaction. Many factors, which must be taken into

account in planning this step, have important effects on hybridization including the composition of the hybridization buffer, probe concentration, and preservation of tissue morphology. Although many studies have investigated the effect of hybridization conditions on in situ hybridization, the basic conditions were reinvestigated to optimize the method for the tissue, i.e. decidua, amnion, chorion, trophoblast and placenta used in subsequent studies in this thesis.

Formamide which is capable of disrupting hydrogen bonds decreases the melting temperature of hybrids by 0.7°C per 1% of formamide for DNA hybrids, and 0.4°C for RNA hybrids, and so allows the use of lower hybridization temperatures resulting in better tissue morphology (Angerer et al., 1987). By keeping the hybridization temperature constant and varying the formamide, the most effective temperature could be determined. Inclusion of dextran sulphate and other polymers accelerates the rate of hybridization by volume exclusion, which increases effective probe concentration. Increased ionic strength (sodium ion concentration) has a stabilizing effect on nucleic acid duplexes. However increasing ionic strength above 0.4 M NaCl has little effect on hybridization or hybrids stability (Meinkoth and Wahl, 1984).

By considering the factors discussed above, the experiment in this study were carried out at 42°C by using a hybridization buffer containing 50% formamide and followed by a series of posthybridization washes at increasing stringencies, i.e. decreasing the salt concentration. All of the conditions used favoured the detection of well matched hybrids.

The appropriate probe concentration for in situ hybridization is that which produces the greatest signal-to-noise ratio. Less probe would result in a decreased signal and more would only increase the background. The desired probe concentration in this study seemed to be 1×10^6 cpm/slide because there was no further increment of the signal when the probe concentration was increased to 1.5×10^6 cpm/slide.

The final protocol of in situ hybridization used in this study was as follows:

Paraffin sections were dewaxed in 2 changes of xylene for 10 min, rehydrated in 100%, 80%, 50% alcohol and rinsed in DEPC water for 5 min prior to the Step 1.

1. The sections were incubated in 0.2 M HCl for 20 min at RT;
2. Transferred to preheated 2X SSC for 10 min at 70°C;
3. Washed in DEPC water for 5 min at RT;
4. Washed in 100 mM Tris/HCl, pH7.5 for 5 min at RT;
5. Digested with 5 µg/ml proteinase K in 100 mM Tris/HCl pH7.5, 2 mM CaCl₂ for 30 min at 37°C;
6. Immersed in 0.2% glycine in PBS for 30 sec to stop the proteinase K action, followed by rinsing in PBS for 5 min;
7. Rinsed in PBS and post fixed in 4% paraformaldehyde for 20 min at RT;
8. Washed in PBS twice for 10 min;
9. Prehybridization: the sections were incubated in 70 µl of prehybridization buffer at 42°C for 1 h;
10. Hybridization: the hybridization was carried out for 18-36 h at 42°C in hybridization buffer containing heat denatured [³⁵S]-PRL-DNA probe at a concentration of 1x10⁶ cpm/slide;
11. Washes: after hybridization, the slides were immersed in 4XSSC for 15 min to remove coverslip, and then washed in the following solutions:

1. 2XSSC for 2 h at RT;
 2. 1XSSC for 2 h at RT;
 3. 0.5XSSC for 1 h at RT;
- Finally, 0.5XSSC for 1 h at 37°C.

12. Following washes, the slides were dehydrated by passing, for three min each, through an alcohol series (50%, 85%) containing 300 mM ammonium acetate to stabilize the hybrids and one change of 100% alcohol. The slides coated with emulsion were then exposed at 4°C for 7 to 14 days before autoradiography.

Part II. Modification of the immunostaining method for immunolocalization of decidual PRL in utero-placental unit

2.2.1. Introduction

The initial intention in this chapter was to use the avidin-biotin immunostaining method set up in our histology laboratory to localize the decidual PRL. However the achievement of optimal specific staining is related to the proper antibody dilution, incubation time and temperature, appropriate tissue fixation, as well as the minimal background staining. These factors affect the quality of immunostaining in a complementary fashion, but vary according to the antigen to be localized and the primary antibody. These conditions were, therefore, investigated to determine the best protocol for the immunolocalization of decidual PRL.

2.2.2. Materials and methods

2.2.2.1. Fixation

The tissue from early and term pregnancies was collected and embedded in paraffin and sectioned exactly as described in Part I. Three cross-linking fixatives were evaluated for proper fixation:

1. 4% neutral buffered formalin at RT for 24 h;
2. 4% paraformaldehyde at RT for 24 h;
3. 2.5% glutaraldehyde at 4°C for 24 h.

After fixation, tissue was pretreated with or without 0.1% (w/v) trypsin (BDH Chemicals Ltd., Poole, England) and 0.5% (v/v) Triton-X 100 before the application of primary antibody (See **Appendix 5** for preparation of immunostaining solutions).

2.2.2.2. Antibodies

The following checkboard was used to determine the optimal dilution of primary and secondary antibodies, as well as incubation time.

Secondary antibody	Primary antibody		Time & temperature (incubation)
1:200	1:100	1:200	30 min at RT
	1:500	1:1000	24 h at 4°C
1:500	1:100	1:200	30 min at RT
	1:500	1:1000	24 h at 4°C

The primary antibody was an anti-human PRL antibody raised in a rabbit (Lot no. 068, Dako Corporation, Santa Barbara, CA, USA) and biotinylated swine anti-rabbit immunoglobulin G (Dako, Lot no. 129) was used as the secondary antibody. The avidin-biotin complex (Dako, Lot no. 067) was used at the concentration recommended by the manufacturer.

2.2.2.3. Background staining

The major problem in performing immunocytochemistry is background staining. The following efforts were taken to decrease background staining according to the main cause of this problem:

1. Suppression of endogenous peroxidase activity by incubating the sections with 3% (v/v) H_2O_2 in 100% methanol for 30 min;
2. Reduction of ionic interaction of tissue with antibody by addition of 0.9% NaCl in 0.05 M Tris/HCl buffer (Tris Buffered Saline; TBS);
3. Involvement of 5% (w/v) bovine serum albumin (BSA) in blocking reagent (normal swine serum 1:4 diluted in TBS);
4. The protein concentration of the normal rabbit serum was adjusted to be the same as that of primary anti serum.

2.2.2.4. Control

Controls of the method and specificity of the antisera were: 1) sections of human anterior pituitary gland as positive and human tonsil as negative controls (tissue sections supplied by Dr K. Grigor, Department of Pathology, Royal Infirmary, Edinburgh); 2) substitution of normal rabbit serum and normal sheep serum for the specific serum; 3) omission of primary antiserum.

2.2.3. Results and discussion

Formaldehyde is the most popular fixative employed for immunocytochemistry study on paraffin-embedded sections. It generally preserves good morphology and maintains the immunoactivity as well provided that tissue is fixed under suitable conditions and for the optimal time (Farmilo and Stead, 1990). Therefore a phosphate buffer with neutral salt was used to make up 4% formaldehyde to maintain tonicity and pH (7.4) in this study to optimize the conditions for tissue fixation. Early and term pregnancy tissues were fixed for 8, 24, or 72 h respectively followed by digestion with or without trypsin. Since formaldehyde forms cross-linkages with basic amino acids, mainly lysine and arginine, there is a relatively low permeability to antibody. However trypsin digestion compensates for the impermeable nature of the fixative by cleaving adjacent to lysine and arginine to promote antibody penetration (Farmilo and Stead, 1990). As illustrated in Plate 2.2.B, without trypsin digestion, no specific PRL staining was localized over the sections after fixed in 4% neutral buffered formalin for 24 h. Staining was seen after addition of trypsin digestion and, of the three different time of fixation, 24 h gave the strongest specific staining and least background (2.2.A). When the tissue was fixed for 72 h which was beyond the optimal fixation time, the antigenic site appeared to be masked probably due to excessive cross-linking of tissue proteins and increased hydrophobicity of the tissue, which consequently resulted in very weak staining as shown in (2.2.C). In contrast insufficient fixation (8 h in this case) made tissue too soft to be sectioned and destroyed the morphological integrity of the specimen. Although PRL staining intensity was slightly less in 4%

paraformaldehyde fixed tissue than that fixed in 4% neutral buffered formalin when applied at same concentration and incubation duration of the primary antibody (2.2. D), it was still used for those studies in this thesis in which in situ hybridization and immunocytochemistry was carried out on serial sections of the same tissue. Because glutaraldehyde is most effective at cross-linking and causes loss of more immunological activity, very weak PRL staining was observed on sections fixed with 2.5% glutaraldehyde (2.2. E).

Apart from trypsin digestion, a further attempt to improve antibody penetration was tried using pretreatment of sections with 0.5% (v/v) Triton-X 100 to remove the lipid across the cell membrane (Hartman, 1973). After Triton-X 100 treatment, the background staining was not increased, while the intensity of staining was improved. Thus pretreatment with Triton-X 100 was adopted as a routine procedure for the immunocytochemistry study in this thesis.

The determination of the optimal antibody dilution and incubation duration is one of the most critical steps in immunocytochemistry study. For each new batch of reagents these parameters were always checked by using the checkboard shown before. A primary antibody dilution between 1:500 and 1:1000 in combination with secondary antibody at a dilution of 1:500 at room temperature for 0.5 h incubation was the optimal protocol for localization of PRL in utero-placental unit (2.2.A). The increase or decrease of antibody concentration or incubation duration enhanced background staining or attenuated specific staining.

Further efforts to improve the quality of immunostaining was designed to decrease the background staining which is the most common problem encountered in immunostaining. Besides including 0.9% NaCl in the Tris buffer to reduce the ionic interaction with opposite net charged antibody (Boenisch, 1990) and adopting normal swine serum as blocking serum, it was found that the addition of 5% BSA in the blocking serum and the diluent of antibody diminished the background staining to the greatest extent (2.2.A and F). In addition, the endogenous peroxidase activity was quenched by using 3% H_2O_2

without exception since the tissue always contained many red blood cells with high endogenous peroxidase activity.

There was no specific staining when normal rabbit serum was substituted for primary antibody, in which the normal rabbit serum was diluted to a protein concentration identical to that in the primary antibody. In addition no positive staining for prolactin was observed in sections of human tonsil. These results confirm that the immunostaining for PRL was specific.

The optimal immunostaining method for PRL in utero-placental unit is shown in following:

Sections were deparaffinised in xylene and brought to alcohol. Unless otherwise specified all the sections were sequentially incubated for various time at room temperature with each of following reagents:

1. 3% (v/v) hydrogen peroxide in methanol to block endogenous peroxidase activity for 30 min;
2. 0.1% (w/v) trypsin and CaCl_2 , pH 7.4, 37°C for 30 min;
3. 25% (v/v) normal swine serum and 5% (w/v) bovine serum albumin (BSA) in 0.05 M Tris/HCl with 0.15 M NaCl, pH 7.6 (TBS), for 30 min;
4. Rabbit anti-hPRL serum (1:500, 1:1000) for 30 min;
5. Biotinylated swine anti-rabbit immunoglobulin G diluted (1:500) in TBS for 30 min;
6. Avidin-biotin complex: 40 μl of each in 5 ml 0.05 M Tris/HCl (pH 7.6) for 30 min.

After each incubation the slides were washed with TBS for 10 min except after incubation with normal swine serum. Hormone localization was determined by detection of peroxidase activity, effected by 5 min incubation of the slides with a freshly prepared solution containing DAB (4 mg/10 ml 0.05 M Tris/HCl to which was added 0.2 ml 3% (v/v) hydrogen peroxide). Sections were counterstained with haematoxylin, dehydrated and mounted in DPX mounting medium (Raymond A. Lamb., North Action, London, NW10 6JL).

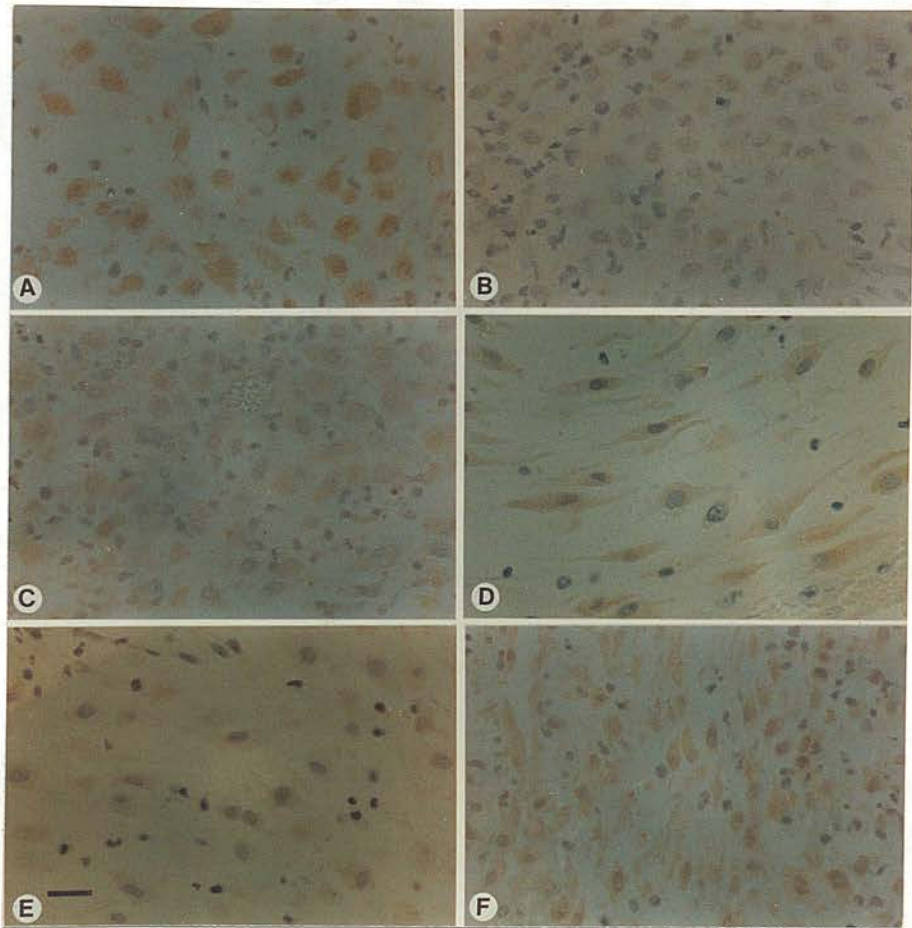


Plate 2.2.

The development of the immunocytochemistry method to localize decidual PRL in utero-placental unit. The scale bar is 20 μ m.

(2.2.A.) Immunolocalization of PRL in human decidua fixed with 4% neutral buffered formalin for 24 h. Predigestion using 0.1 % (w/v) trypsin was performed on the sections and the primary and secondary antibodies were both used at a dilution of 1:500. 5% BSA was added to the blocking serum.

(2.2.B.) Immunolocalization of PRL in human decidua performed under the same condition as 2.1.A., but without trypsin digestion. Note very faint PRL immunostaining observed over the decidual cells.

(2.2.C.) Immunolocalization of PRL in human decidua fixed in 4% neutral buffered formalin for 72 h. Note apparently decreased staining intensity of PRL over the decidual cells compared with 2.2. A.

(2.2.D.) Immunolocalization of PRL in human decidua fixed in 4% paraformaldehyde for 24 h. Note slightly reduced staining intensity of PRL in the decidual cells compared with 2.2.A.

(2.2.E.) Immunolocalization of PRL in human decidua fixed in 2.5% glutaraldehyde for 24 h. Note very faint PRL immunostaining observed over the decidual cells.

(2.2.F.) Immunolocalization of PRL in human decidua performed under same conditions as 2.2.A, but without involvement of 5% BSA in the blocking serum. Note much higher nonspecific background staining over the section than that in 2.2.A.

Part III. Localization of the sites of synthesis and action of prolactin by immunocytochemistry and in situ hybridization within the human Utero-placental unit

2.3.1. Introduction

Many studies support the concept that the high concentration of PRL found in amniotic fluid during human pregnancy originates from the decidualized endometrium (Bigazzi et al., 1979b; Healy et al., 1979). This PRL appears to be identical to pituitary PRL (Golander et al., 1978). However the definitive cell type which is responsible for PRL production in decidua has not been localized.

Several groups have attempted to isolate these PRL producing cells by a number of different methods, including isopycnic centrifugation (Markoff et al., 1983a), immunocytochemistry (Braverman et al., 1984) and fibroblast-free decidual cell culture (Hochner-Celnikier et al., 1984). Results from these studies have suggested that the PRL-producing cells are morphologically similar to decidual cells.

While these studies suggest that only the decidua is the source of PRL in the utero-placental unit, high concentrations of PRL have also been localised by immunocytochemistry in the amnion (Healy et al., 1977) and the trophoblast (Al-Timimi and Fox, 1986). It is not clear whether this PRL is synthesized in these PRL immunopositive cells or whether it is sequestered from the extracellular space. In addition, the role of decidual PRL within the fetu-utero-placental unit is still obscure.

The aim of the study in this part of the thesis was to determine the specific cell type which synthesizes PRL together with a precise localization of the tissues which accumulates PRL by using the combined methods of immunocytochemistry and in situ hybridization as developed and described in the previous parts of this Chapter. Thus a positive identification of the site of synthesis of PRL together with a precise localization of the cell types which accumulates PRL should provide evidence for the potential site of action of decidual PRL. In

addition, localization of the specific PRL-producing cells is essential for further determination of the relevant regulators of decidual PRL synthesis and release.

2.3.2. Materials and methods

Immunocytochemistry and in situ hybridization to localize PRL and its mRNA in utero-placental unit were performed as described in Part I and II of this Chapter. Tissues from three patients at both early (8-11 weeks) and term pregnancies (37-40 weeks) were used in this study.

2.3.2.1. Northern blot Analysis

The localization of decidual PRL mRNA was cross-checked by Northern blot analysis which was performed according to the routine method used in our laboratory (Preparation of solutions for Northern blot analysis is shown in **Appendix 6**).

A. RNA extraction procedure

Total RNA was extracted from the frozen term decidua according to the method of Chomczynski and Sacchi (1987). Ovine pituitary RNA was also extracted for comparison with the decidual PRL mRNA. This pituitary was collected from an anoestrous ewe within 3 min of death induced by an overdose of sodium pentobarbitone.

The collected tissue samples stored in liquid nitrogen until RNA extraction were placed onto aluminium foil on dry ice, weighed and dropped into solution D (1 ml solution D/100 mg tissue), which had been chilled on ice for no more than 5 min, and then homogenized immediately using a polytron homogenizer for 30 sec at speed 8. The homogenate was transferred to a sterile polypropylene tube (Falcon 12 ml) and the following solutions were sequentially added (quantities/1 ml solution D):

0.1 ml sodium acetate (2 M, pH 4.0)

1 ml water saturated phenol

0.2 ml chloroform/isoamyl alcohol (49:1)

The final mixture was shaken vigorously for at least 10 sec, and centrifuged at 10,000 g for 20 min at 4°C. The aqueous phase containing RNA was removed to a new tube, mixed with an equal volume of cold isopropanol which was stored in a -20°C freezer and added immediately after removal from freezer, and placed at -20°C for at least 1 h to precipitate RNA. The solution was then centrifuged at 10,000 g for 20 min at 4°C to pellet RNA. After dissolving the pelleted RNA in 1 ml solution D, the RNA was reprecipitated as described above. The pellet was dried under nitrogen and then redissolved in 200 µl pure water containing 0.1% (w/v) sodium dodecyl sulphate (SDS) by warming up to 65°C for 10 min, and then stored at -70°C. The concentration and purity of RNA were determined at 260 nM and 280 nM in a Spectrophotometer. The ratio of readings at 260/280 gives the purity and the concentration of RNA is determined from the 260 nM reading (40 µg/ml RNA=1.0)

B. Separation of RNA and hybridization

If the volume of the samples containing the required amount of RNA (15 µg) was more or less than 5 µl, the samples were concentrated by precipitating with ethanol or diluted with sterile water to bring the required concentration of RNA to 5 µl.

RNA (15 µg) was denatured in 16 µl of sample buffer containing 17.4% formaldehyde, 50% formamide, 20 mM 3-[N-morpholino]propanesulphonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA at 60°C for 5 min. RNA was then fractionated on a 1.5% agarose/0.66 M formaldehyde mini-gel running at 100 voltage for 2.5 h and transferred to a nylon membrane (Hybond-N, Amersham, UK). After air-drying and crosslinking with UV light for 2 min, the membrane was prehybridized at 65°C for 5 h with 20 ml hybridization buffer containing 5XSSC (1XSSC being 0.03 M NaCl, 0.003 M sodium citrate), 0.1% (w/v) SDS, 0.1% (w/v) sodium pyrophosphate, 0.05% (w/v) ficoll 400, 0.05% (w/v) polyvinylpyrrolidone (PVP), 0.05% (w/v) BSA and 100 µg/ml sheared herring sperm DNA. Hybridization was carried out in 20 ml fresh hybridization buffer containing $1-2 \times 10^6$ cpm/ml [32 P]-

radiolabelled cDNA probe (See **Appendix 3** for cDNA probe labelling method) overnight at 65°C. After washing twice in 40 mM sodium phosphate (pH 7.2), 1 mM EDTA and 1% SDS at 65°C for 1 h, the blot was exposed to Kodak X-OMAT AR film at -70°C with two intensifying screens for 1- 4 days.

2.3.3. Results

2.3.3.1. Northern Blot Analysis

Hybridization occurring between the [³²P]-cDNA PRL probe, and mRNA from ovine pituitary (lanes 1 to 3) and two separate preparations of term decidua (lanes 4 to 5) are shown in Plate 2.3. The mRNA for the human decidual PRL appeared to be larger than that of the ovine pituitary PRL mRNA.

2.3.3.2. Immunocytochemistry and in situ hybridization

PRL immunocytochemical staining and in situ localization of PRL mRNA in the utero-placental unit in early and term pregnancy tissue is illustrated in Plate 2.4.

A. Early Pregnancy

Specific PRL staining was seen both in the trophoblast (2.4.A) and decidua (2.4.C). The staining in the decidua was more intense, and was apparent not only in the decidualized cells but was also in endometrial glands. The immunostaining appeared to be uniform throughout the cytoplasm of all positive staining cells. The in situ hybridizations revealed that all of the silver grains were only associated with decidualized cells, whereas no specific grains were observed over the endometrial glands and trophoblast (2.4.D and 2.4.B).

B. Term pregnancy

Although PRL was detected in cells in the amnion (2.4.E), syncytio- and cytotrophoblast of the placenta (2.4.G) and decidua (2.4.I), it was not

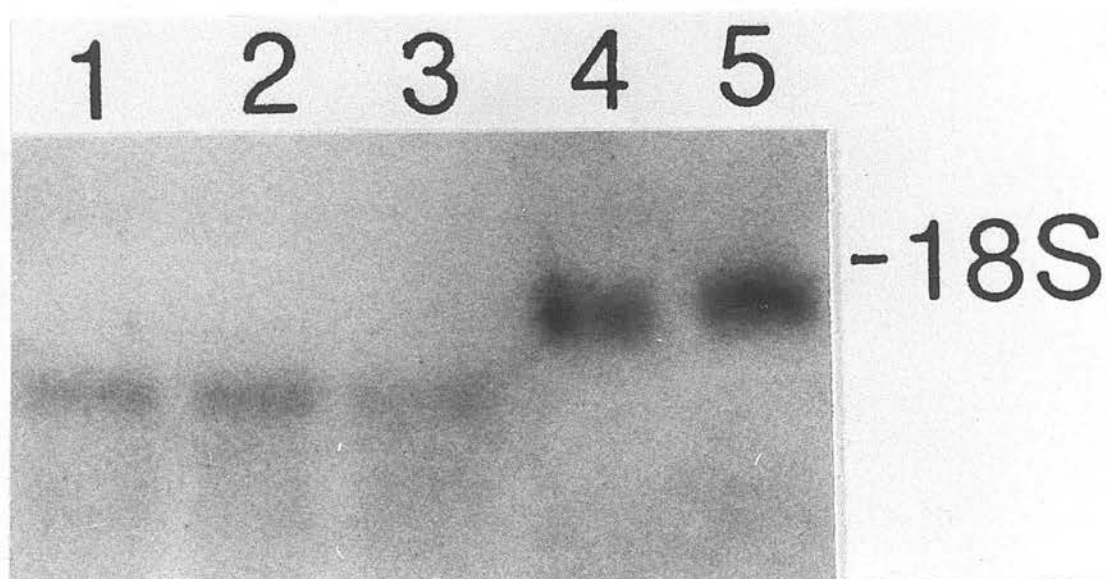


Plate 2.3. Autoradiograph of a Northern blot showing the hybridization of [^{32}P]-hPRL cDNA with RNA extracted from ovine pituitary (lanes 1 to 3; 5 μg , 1 μg and 0.5 μg respectively) and from human term decidua (lanes 4 and 5; 15 μg). The position of the 18S ribosomal RNA is shown on the right.

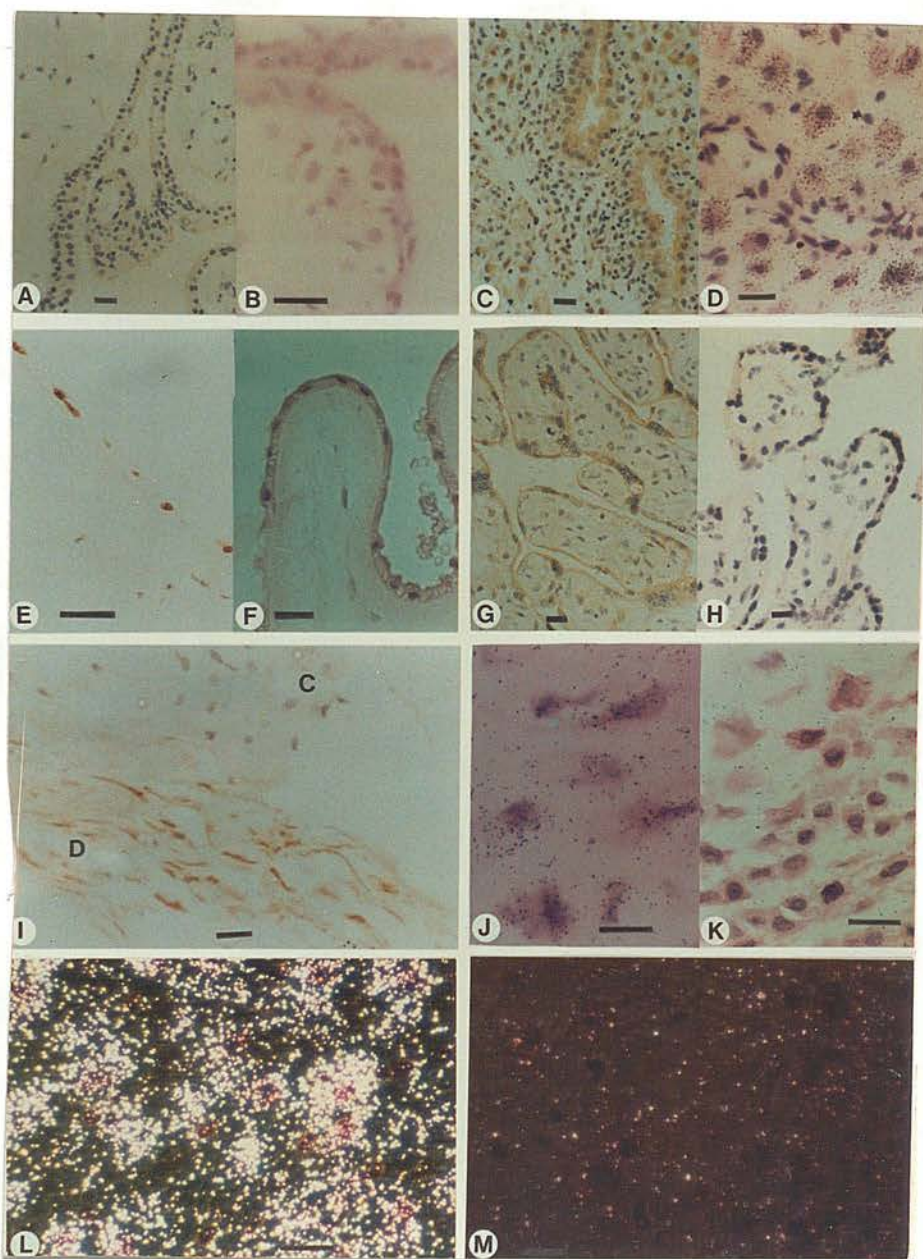


Plate 2.4.

Immunocytochemical staining of PRL and in situ localization of PRL mRNA in human utero-placental tissues from early and term pregnancies. The scale bar is 20 μ m.

- (2.4.A) Immunostaining for PRL in early trophoblast. Note faint but positive staining for PRL.
- (2.4.B) In situ localisation for PRL in early trophoblast. Grains are not readily apparent indicating the absence of specific localization of probe to tissue.
- (2.4.C) Immunostaining for PRL in early decidua. Note intense specific staining for PRL in decidualized cells and endometrial glands.
- (2.4.D) In situ localization for PRL in early decidua. Note abundant black grains over decidualized cells only.
- (2.4.E) Immunostaining for PRL in term amnion. Note positive staining for PRL.
- (2.4.F) In situ localization for PRL in term amnion. Note only background grains present.
- (2.4.G) Immunostaining for PRL in term placenta. Positive staining for PRL again seen.
- (2.4.H) In situ localization for PRL in term placenta. Note presence of background grains only.
- (2.4.I) Immunostaining for PRL in term decidua (D) and chorion (C). Note intense positive staining for PRL in the decidua, but not in the chorion.
- (2.4.J) In situ localization for PRL in term decidua. Note abundant black grains over decidual cells.
- (2.4.K) In situ localization for PRL in term chorion. Note only background grains present.
- (2.4.L) Dark-field photomicrograph of in situ localization for PRL in early decidua. Note abundant white grains.
- (2.4.M) Dark-field photomicrograph of in situ localization for PRL in early decidua treated with RNase. Note only background white grains.



found in the chorion (2.4.I). As in early pregnancy, the only cell types containing PRL mRNA was the decidualized cells (2.4.J) and no PRL mRNA was identified in amnion (2.4.F), in chorion (2.4.K) and placenta (2.4.H).

Pretreatment of tissue sections with RNase before hybridization completely prevented specific labelling of decidualized cells by the [^{35}S] labelled probe (2.4.M), confirming that the labelled probe had hybridized specifically with PRL mRNA (2.4.L).

2.3.4. Discussion

This is the first study to localise the specific cell type for synthesis of PRL by co-localization PRL and its mRNA within the utero-placental unit. Using in situ hybridization, we have identified the mRNA encoding for PRL within decidualized cells. No other cell types within the utero-placental unit in early or term pregnant tissue were found to contain PRL mRNA. While some previous studies identified only the placenta as the source of PRL (Kasai et al., 1982; Al-Timimi and Fox, 1986), this is not confirmed in this study and the present results are in accord with other reports (Pileri et al., 1982; Bryant-Greenwood et al., 1987) identifying PRL in amnion, trophoblast and decidua. However, we could not confirm a further report identifying PRL in the chorion (Frame et al., 1979b), but showed conclusively that PRL is present in both early and term trophoblast in contrast to a previous report (Bryant-Greenwood et al, 1987).

The synthesis and secretion of PRL by decidualized endometrium at term was first shown by Riddick and Kusmik (1977). This finding was then extended to early pregnant endometrial tissue and uterine endometrium obtained from ectopic tubal pregnancies (Maslar et al., 1980). This latter observation suggests that the presence of cells of fetal origin within the uterus is not essential for the production of PRL by the uterine endometrium. Furthermore, Daly et al. (1983a) using an in vitro system showed that proliferative endometrium obtained during the menstrual cycle underwent progesterone-dependent differentiation and synthesized PRL. The initiation of PRL secretion in vitro

corresponded to the onset of histologically apparent decidualization. In addition, when morphological de-decidualization of decidual cells was induced by blocking progesterone action in vivo with the anti progesterone, mifepristone, decidual PRL synthesis was shown to be suppressed (Chapter 4). These results suggested that PRL production is entirely dependent on the decidualization of endometrium.

The present study is the first to localize PRL mRNA within the decidua by in situ hybridization. The use of a human pituitary PRL cDNA probe for in situ hybridization was validated by Northern hybridization, showing that the probe hybridized to a single RNA species. It appeared that the decidual PRL mRNA was longer than that of the ovine pituitary PRL mRNA.

However an early study using Northern blot analysis has reported that the decidual PRL mRNA was of a similar size to ovine pituitary PRL mRNA (Clements et al., 1983). Further support for the concept that decidual PRL gene structure might be similar to that of pituitary PRL was obtained when it was shown that cDNA synthesized from the decidual PRL mRNA had almost precisely the same sequence as pituitary-derived PRL cDNA, suggesting that only one copy of the PRL gene is present in the haploid genome and the same PRL gene is expressed in both pituitary and decidua (Takahashi et al., 1984).

In contrast to those reports that the PRL protein coding sequence in decidua was identical to that in pituitary, it has now been reported that the decidual PRL mRNA is about 150 nucleotides larger than the human pituitary transcript, in accord with the present study, due to an elongation of the mRNA located in the 5' untranslated region of the gene (Gellersen et al., 1989; DiMattia et al., 1990; Hiraoka et al., 1991) implying a different transcription initiation site in decidual PRL gene from the PRL gene in the pituitary. These results might underlie an important mechanism which specifically controls PRL gene expression in decidua.

The results of the present study strongly suggest that only decidualized cells are capable of PRL biosynthesis. Cells of fetal origin, namely

amnion and trophoblast, only accumulate PRL. It may be anticipated that the sites of PRL action may be the amnion and trophoblast where PRL receptors have been identified (Healy et al., 1982; 1985; McCoshen et al., 1982b) and amniotic fluid, a compartment into which decidual PRL is secreted. Indeed, studies have suggested that decidual PRL may play a role in maintaining amniotic fluid volume (Healy et al., 1982; 1985; McCoshen et al., 1982b), in fetal lung maturation (Johnson et al., 1985) and possibly in modulating fetal membrane prostaglandin production (Tyson et al., 1985) to control parturition (Healy et al., 1984).

In summary, these results identify conclusively for the first time that decidual cells within the endometrium are the only major source of PRL synthesis. The fact that other cell types within the trophoblast and the amnion contain PRL suggests that it may play an important paracrine role within the utero-placental unit.

In this chapter, the relationship between decidualization and PRL production at different stage of human pregnancy was studied at the level of synthesis and secretion by means of in situ hybridization and Northern blot to reveal the PRL mRNA level, immunocytochemistry to examine PRL content inside the decidual cells as well as radioimmunoassay to measure PRL output in amniotic fluid.

3.2 Materials and Methods

Immunocytochemistry, in situ hybridization and Northern blot analysis were performed exactly as described in Chapter 2. Samples from two patients in early and late pregnancy were used.

CHAPTER 3

The Relationship between the Decidualization and PRL Production at the Different Stages of Human Pregnancy

3.1. Introduction

It is clear now that the high level of PRL in amniotic fluid is specifically produced by the decidual cells. However the control of decidual PRL secretion is still poorly understood. Previous studies have suggested that the decidualization of the stromal cells in the endometrium is essential for the initiation of PRL production (Maslar and Riddick, 1979; Daly et al., 1983a). Because progesterone is the main hormone involved in differentiating the oestrogen-primed stromal cells into decidual cells, the regulatory effect of both sex steroids on PRL production in the endometrium of normal menstrual cycle has been extensively studied. However, their potential role in PRL production during pregnancy and the relationship between decidualization and PRL production throughout pregnancy remain obscure. Furthermore most studies have not distinguished between regulation of de novo synthesis and regulation of secretion of PRL. Unlike pituitary PRL, decidual PRL is not packaged into secretory granules (Handwerger et al., 1984), so it is important to examine the regulation of PRL at the level of synthesis.

In this chapter, the relationship between decidualization and PRL production at different stage of human pregnancy was studied at the level of synthesis and secretion by means of in situ hybridization and Northern blot to reveal the PRL mRNA level, immunocytochemistry to examine PRL content inside the decidual cells as well as radioimmunoassay to measure PRL output in amniotic fluid.

3.2. Materials and methods

Immunocytochemistry, in situ hybridization and Northern blot analysis were performed exactly as described in Chapter 2. Tissue from two patients in each stage of early, mid and term pregnancy was

used in this study. Tissue fixed in 4% paraformaldehyde was used for both immunostaining and in situ hybridization. Eight serial sections from each block (one patient) were cut and floated onto the previously cleaned and poly-L-lysine coated slides for in situ hybridization and immunocytochemistry. Amniotic fluid samples were collected at amniocentesis by Dr. W. Ledger, Department of Obstetrics and Gynaecology, University of Edinburgh, in 3 early, 14 mid and 16 term pregnancies and PRL was assayed using a two-site immunoradiometric assay (IRMA).

3.2.1. Quantitative analysis of in situ hybridization

All in situ hybridization sections were counterstained with haematoxylin and eosin. Once dehydrated and mounted, sections were examined and photographed using an Olympus photomicroscope under both light and dark field.

In situ hybridization signals from each stage of pregnancy were quantified with a computer-assisted image analysis system. Five areas from each section were randomly chosen for grain counts. This system consisted of a Charge-Coupled Device camera, microscope, and camera monitor linked to an IBM PC/AT microcomputer. The software program (Cue-2) for densitometry was provided by Olympus Optical Company. An optimum threshold and filter combination were set to select only the silver grains. Because in some cells at different stages of pregnancy the density of grains was large, these grains collected into a single large area within the cell preventing accurate counts of individual grains. Therefore, the grain area per cell from five areas per section was calculated as a mean. By combining the means obtained from two sections, an overall mean representing one patient was averaged ($n=1$).

3.2.2. PRL radioimmunoassay procedure

PRL in the amniotic fluid was assayed using a two-site immunoradiometric assay (IRMA) as described in the following:

Amniotic fluid samples (1:10 diluted in assay buffer defined as below) were assayed in duplicate in a volume of 450 μ l. 50 μ l of standard or samples were added to 400 μ l assay buffer (1% (w/v) BSA (Sigma RIA grade, fraction V) in 0.05 M phosphate buffer plus 5% (v/v) Tween 20, pH 7.4). 50 μ l of a sheep anti-prolactin linked to a solid phase was added to all tubes except total counts and incubated with the sample or standard buffer at room temperature on a rotary mixer for 3 h. Following washing twice with washing buffer (0.05 M phosphate buffer with 5% (v/v) Tween 20), 50 μ l of the radioiodinated mouse monoclonal anti-prolactin antibody, diluted in assay buffer to give 100,000 counts/50 μ l/100 seconds, and 400 μ l of assay buffer, were added to all tubes and the reaction continued overnight on a rotary mixer, followed by washing and centrifugation twice with washing buffer, and then counting. The detection limit of this assay was 50 mU/L. Intra- and inter- coefficients of variation were 2.5% and 12% respectively.

All the reagents, i.e. standards, solid phase, and radioiodinated second antibody, were purchased from NETRIA (NETRIA, St Bartholomews Hospital, London, U.K.).

3.2.3. Data reduction

The two-site PRL (IRMA) was counted in a Multigamma Counter (1261 Multigamma, LKB Wallac OY, Turku, Finland), from which the output of the raw counts was recorded by a digital data logger (Data grabber, Mutek, Box, Wiltshire) onto a 3.5 inch floppy disc. The data were then transferred from the data grabber disc into an Apple Macintosh computer where raw counts were analysed by the AssayZap universal assay calculator program (Elsevier, Biosoft, UK). Briefly, a four-parameter weighted-regression model of displacement curve was utilized for this program. This begins with the two-parameter Logit-log model as a first estimate but then adjusts the asymptotes, slope and point of inflection (the four parameters) of model curve reiteratively, in order to give more weight to the standard points with smaller variance, until optimum squares fit is obtained. Assayzap also preserves the previous information of assay history in order to compare with previous standard curves, quality control and binding level.

3.2.4. Statistical analysis

All statistical analyses were performed on a Apple Macintosh computer. The linear regression and correlation between the cell size and grain area were conducted using Statworks (Data Metrics Inc., USA) program. The difference in PRL concentration of the different stage of pregnancy in amniotic fluid was assessed by analysis of variance using a statistics program written for the Apple Macintosh computer (CLR Anova, Clear Lake Research, Houston, TX, USA).

3.3. Results

3.3.1. Immunocytochemistry, in situ hybridization and Northern Blot

PRL immunostaining and PRL mRNA localization by in situ hybridization are shown in Plate 3.1. In early pregnancy decidualization was not uniform throughout the specimen, and the intensity of immuno- and in situ localization of PRL and its mRNA corresponded very well with the decidualization, i.e. the better decidualized the cells, the more PRL mRNA and PRL were contained in the cells as shown in Plate 3.1. B, C, E and F. Plate 3.1.A and D are the dark field photomicrograph of in situ localization of PRL mRNA in early pregnancy decidua. From early to mid pregnancy, more stromal cells were decidualized indicated by the enlargement of the cell size and nuclei. The grain density and the content of PRL in each individual cell were dramatically increased as illustrated in Plate 3.1. G (dark field), H and I. However, when the decidualization still progressed in term decidua, there was no obvious increment in the average grain area and PRL content in term decidual cells (Plate 3.1. J (dark field), K and L). Both grain area and PRL content were still much higher than that in early pregnancy. This was reconfirmed by Northern Blot analysis (Plate 3.2.), in which the hybridization signals in term pregnancy decidua were obviously stronger than that in early pregnancy decidua.

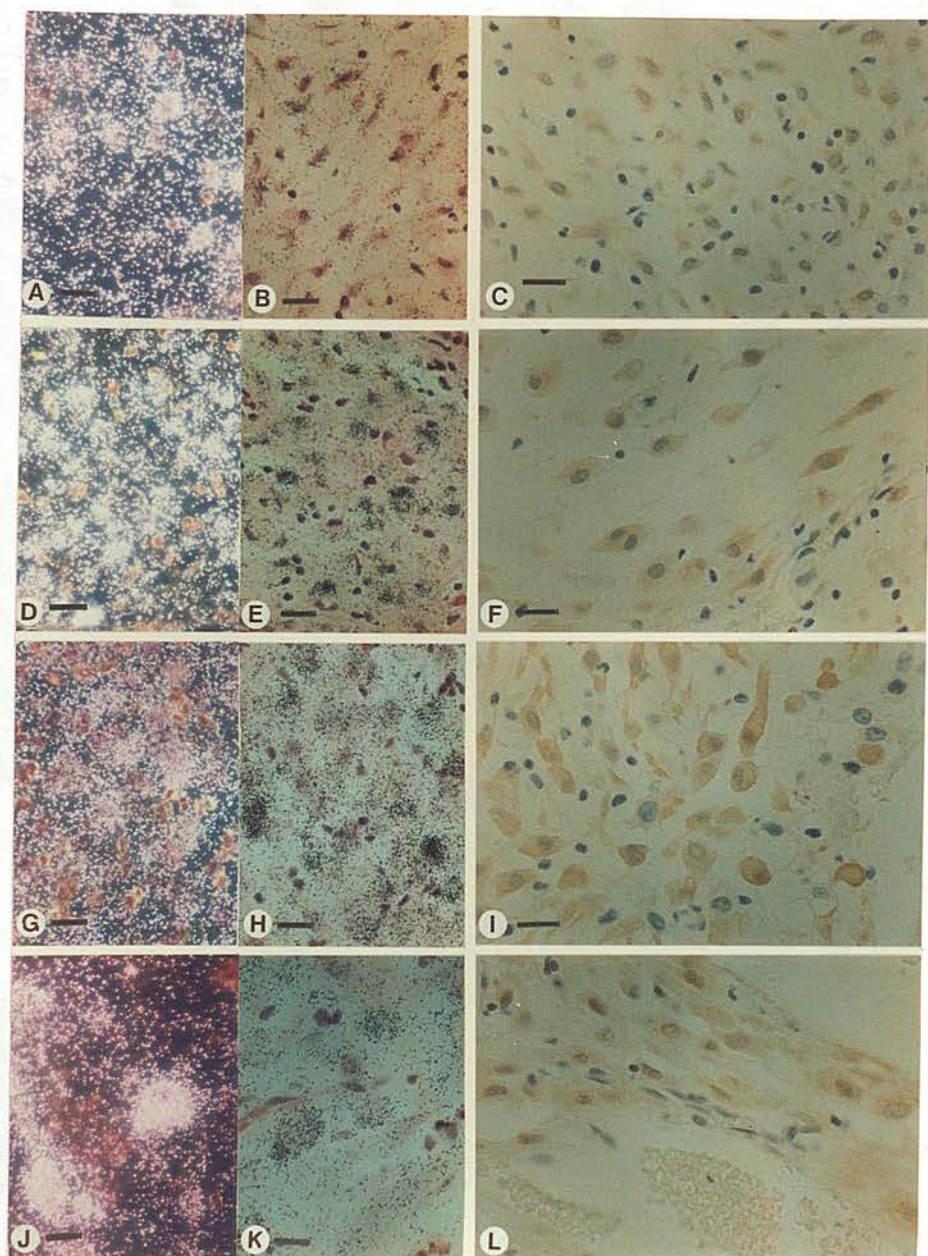


Plate 3.1.

In situ localization of PRL mRNA and immunocytochemical staining of PRL in human decidua from early, mid and term pregnancies. The scale bar is 20 μ m.

(3.1.A & B) Dark field (A) and light field (B) micrographs of in situ localization of PRL mRNA in the decidua of early pregnancy. Note the decidual cells are still like fibroblast cells and a low level of black grains overlay the cytoplasm of the cells.

(3.1.C) Immunolocalization of PRL in the decidua of early pregnancy. Note the shape of the decidual cells is same as that observed in 3.1.B and very faint staining for PRL in the cytoplasm of the cells.

(3.1.D & E) Dark field (D) and light field (E) micrographs of in situ localization of PRL mRNA in the decidua of early pregnancy. The picture was taken from the same section as shown in 3.1. A, B and C, but from a different area. Note the increase in the cell size indicating the more decidualization and apparently increased level of black grains over the decidual cells.

(3.1.F) Immunolocalization of PRL in the decidua of early pregnancy from the same section as 3.1.C, but from a different area. Note the decidual cells become more decidualized indicated by the enlargement in cell size with obviously increased intensity of the immunostaining.

(3.1.G & H) Dark field (G) and light field (H) micrographs of in situ localization of PRL mRNA in the decidua of mid pregnancy. Note the decidual cells have increased dramatically in size and much higher level of black grains overlays the individual cells than that in early pregnancy. The degree of decidualization is uniform over the whole section.

Continued....

- (3.1.I) Immunolocalization of PRL in the decidua of mid pregnancy. Note the increase in cell size with more intense staining of PRL in the cytoplasm.
- (3.1.J & K) Dark field (J) and light field (K) micrograph of in situ localization of PRL mRNA in the decidua of term pregnancy. Note decidual cells continue to increase in the cell size, but without an apparent increase in the level of black grains in the individual cells.
- (3.1.L) Immunolocalization of PRL in the decidua of term pregnancy. Note the intensity of immunostaining of PRL in the decidual cells is similar to that in the mid pregnancy, although the cells continue to increase in size.

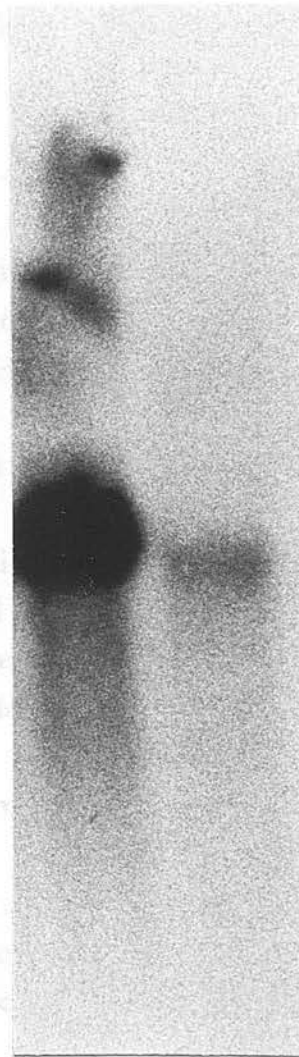


Plate 3.2. Autoradiograph of a Northern blot displaying the hybridization of [32 P]-hPRL cDNA with RNA (15 μ g/lane) extracted from one term decidua (lanes 1) and one early decidua (lane 2). The position of the 18S and 28S ribosomal RNA is shown on the right.

The quantitative results of in situ hybridization are presented in Fig.3.1. The average cell size (3.1.A) and grain area (3.1.B) from early to mid pregnancy both increased dramatically. However, cell size continued to increase in term decidua, PRL mRNA concentration in the individual cells did not increase further from mid to term pregnancy. All these quantitative results were in good agreement with the results of the morphological overview.

The percentage of cells containing PRL mRNA localized by in situ hybridization at the different stages of pregnancy is demonstrated in Fig. 3.1. C. Again, the number of positive cells increased dramatically from early to mid pregnancy (from 9.8% to 47.3%), and then slightly increased towards term pregnancy (57.8%).

3.3.2. Correlation between cell size and its mRNA

There was a significant correlation ($p < 0.0001$) between the cell size and PRL mRNA level in early, mid and term decidua as shown in Fig. 3.2. A, B and C, although no additional increment was noted in PRL mRNA level in term decidua.

3.3.3. PRL concentration in amniotic fluid

The PRL concentration in amniotic fluid in early, mid and term pregnancy is shown in Fig. 3.3. PRL in amniotic fluid increased dramatically ($p < 0.01$) from early ($12.7 \text{ mU/L} \pm 3.4 \text{ sem}$) to mid pregnancy ($2755 \text{ mU/L} \pm 1269 \text{ sem}$) and then decreased significantly ($p < 0.01$) towards term pregnancy ($668 \text{ mU/L} \pm 423 \text{ sem}$), which was still significantly higher than that in early pregnancy ($p < 0.05$).

3.4. Discussion

These results clearly show that PRL production in human decidua is entirely dependent on decidualization in vivo. This confirms the previous in vitro studies that the commencement of decidual PRL synthesis coincided with the onset of morphological changes indicative of decidualization, and the capacity of the endometrium to produce PRL

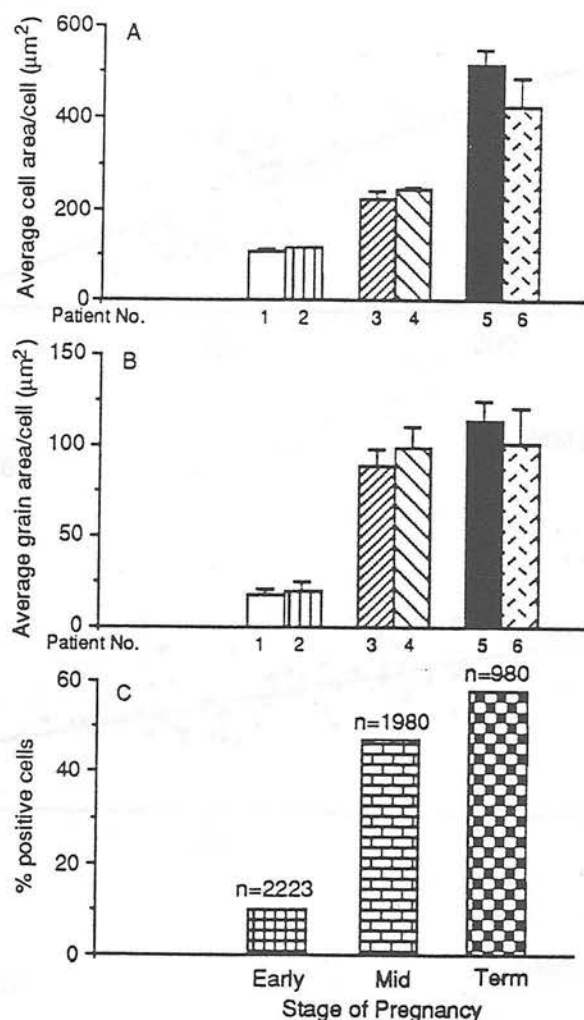


Fig. 3.1. Changes in average cell area (A), average grain area per cell (B) and percentage of cells showing PRL gene expression by in situ hybridization (C) in samples of decidualized endometrium from early (n=2), mid (n=2) and term (n=2) pregnancy. Note the dramatically increase in the average cell size and PRL mRNA levels represented by grain area in the individual cells from early to mid pregnancy, while from mid to term pregnancy only the continuance increase in cell size was observed with maintenance of grain area in the individual cells. The number of positive in situ cells increases, again, markedly from early to mid pregnancy and then slightly increases towards the term pregnancy.

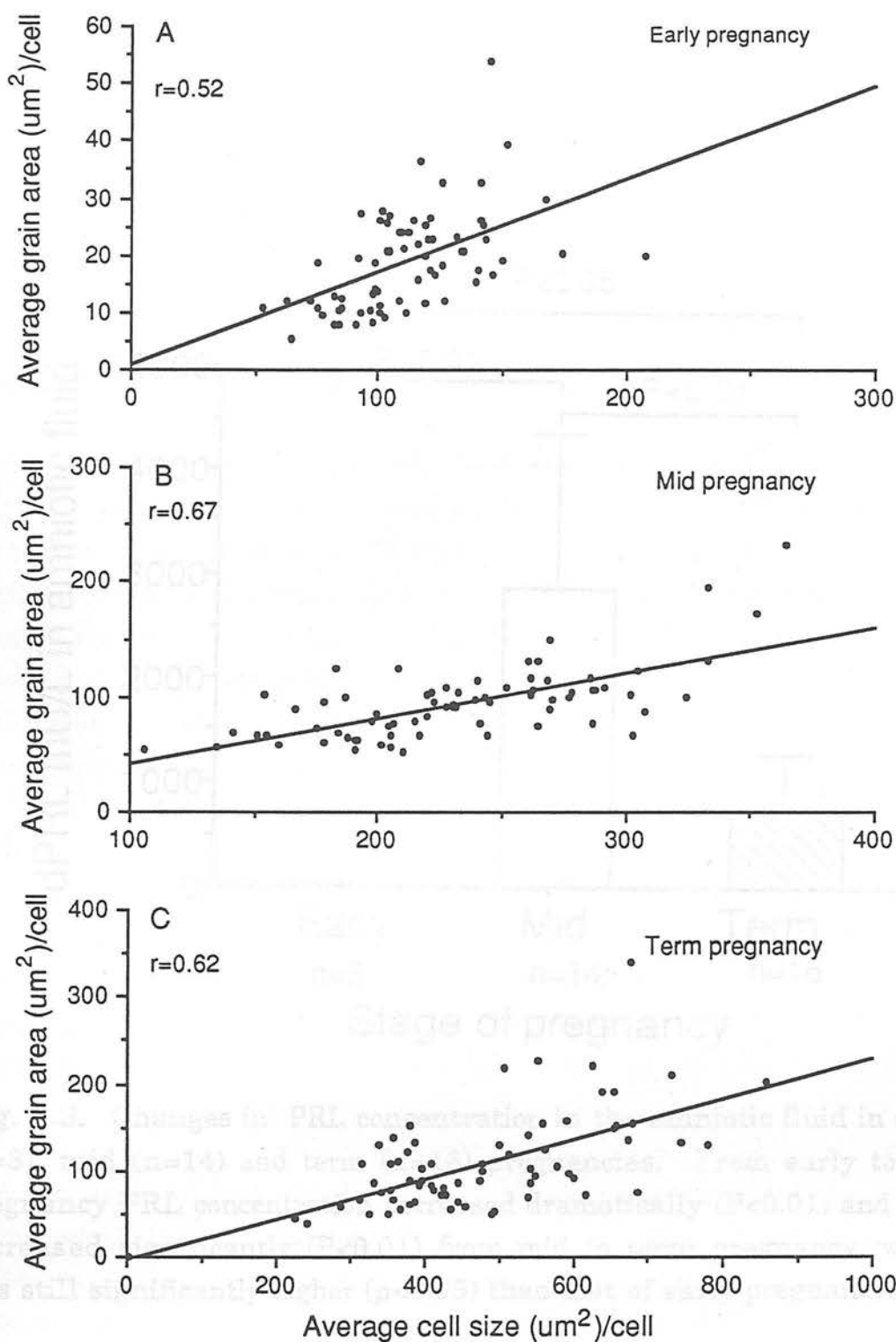


Fig. 3.2. The relationship between the decidual cell size and PRL mRNA level is demonstrated in A (early pregnancy), B (mid pregnancy) and C (term pregnancy). The close correlation between the decidual cell size and the grain area in the individual cells exists in early ($r=0.52$), mid ($r=0.67$) and term ($r=0.62$) pregnancies ($P<0.0001$).

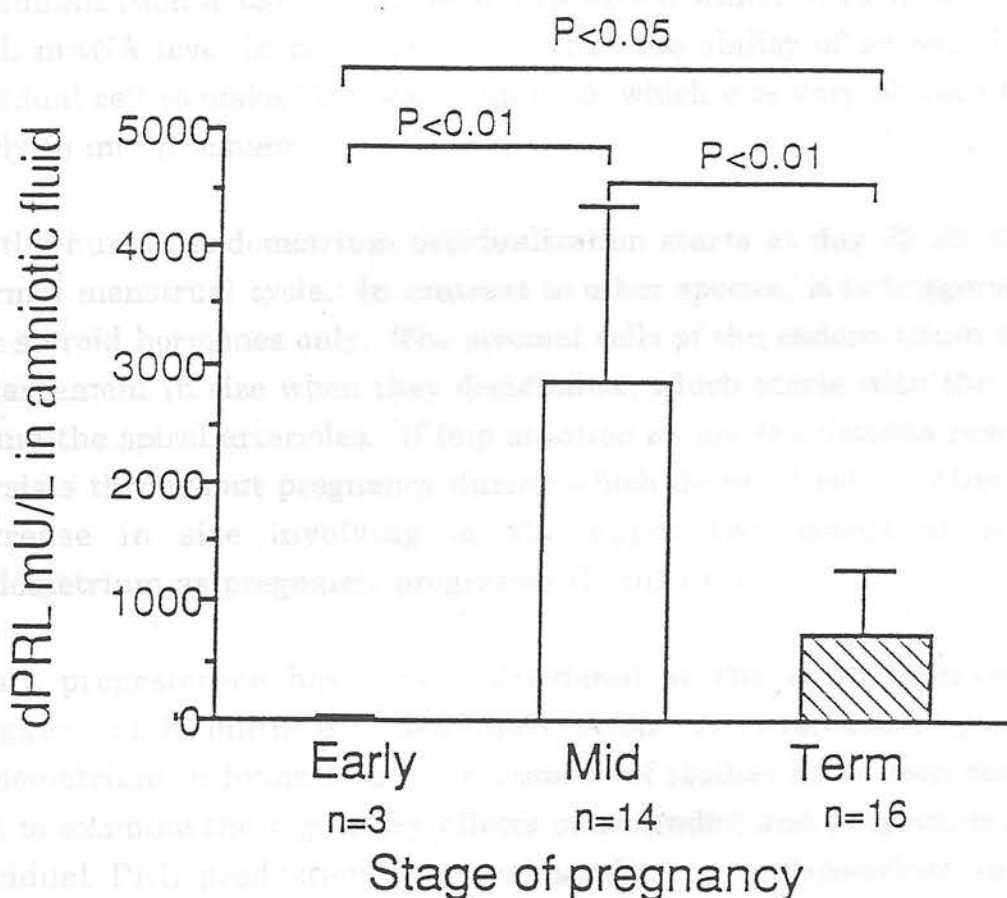


Fig. 3.3. Changes in PRL concentration in the amniotic fluid in early (n=3), mid (n=14) and term (n=16) pregnancies. From early to mid pregnancy PRL concentration increased dramatically ($P<0.01$) and then decreased significantly ($P<0.01$) from mid to term pregnancy, which was still significantly higher ($p<0.05$) than that of early pregnancy.

in vitro increased when decidualization spread throughout the endometrial stroma (Maslar and Riddick, 1979; Maslar et al., 1980). However the present results extend these previous study by demonstrating decidualization has affected not only on PRL secretion but also on PRL gene expression in decidual cells, that is decidualization acted on PRL gene expression which in turn increased PRL mRNA level in decidual cells. Thus the ability of an individual decidual cell to make PRL was magnified, which was very obvious from early to mid pregnancy.

In the human endometrium decidualization starts at day 22-23 of the normal menstrual cycle. In contrast to other species, it is triggered by sex steroid hormones only. The stromal cells of the endometrium show enlargement in size when they decidualize, which starts with the cells round the spiral arterioles. If implantation occurs the decidua reaction persists throughout pregnancy during which decidual cells continue to increase in size involving in the upper two thirds of whole endometrium as pregnancy progresses (Finn, 1977).

Since progesterone has been considered as the main hormone to trigger and maintain decidualization of oestrogen primed endometrium in human, a large number of studies have been carried out to examine the regulatory effects of oestradiol and progesterone on decidual PRL production. Several studies on endometrium in the normal menstrual cycle demonstrated that progesterone alone was capable of generating the decidualization of the stromal cells and stimulating and maintaining synthesis of decidual PRL (Daly et al., 1983a; Huang et al., 1987; Ying et al., 1988; Randolph et al., 1990). However this is not the case in either early or term human pregnancy, because several studies could not confirm that progesterone alone was able to stimulate PRL production in pregnancy decidua upon in vitro culture system (Rosenberg and Bhatnagar, 1984; Bischof et al., 1986; Ren et al., 1990). Therefore, it is unlikely that the effect of progesterone alone can explain the dramatic increase of PRL produced by human decidua during pregnancy.

The direct role of oestradiol alone in decidualization and consequent

PRL production by decidualized cells is less defined even in normal menstrual cycle, but it is generally acceptable that oestrogen priming enhances the decidualization induced by progesterone (Irwin et al., 1989). Although a number of studies have shown that progesterone alone was capable of inducing decidualization and PRL production in vitro as discussed above, these experiments were performed either on proliferative phase or on early luteal phase endometrium which already had oestrogen priming in vivo. Thus oestrogen might be indirectly involved in the control of decidualization and subsequent PRL production in the endometrium.

As the stimulating effect of progesterone on PRL production was attenuated during pregnancy according to the results of in vitro culture as discussed above, it would be logical to ask whether progesterone is still a regulator of decidual PRL production during pregnancy. Since decidualization keeps progressing throughout pregnancy, to reveal the relationship between decidualization and PRL production in decidua throughout pregnancy will indirectly unveil the regulatory effect of the sex steroid hormones on decidual PRL production during pregnancy.

The present study is the first to examine PRL gene expression and the translated product of PRL gene in the decidua from the different stages of pregnancy, representing the different degree of decidualization observed throughout pregnancy. The results clearly show that PRL mRNA level, revealed by in situ and Northern hybridization, and PRL content inside individual cell, exhibited by immunocytochemistry, and PRL output in amniotic fluid, measured by radioimmunoassay, were in good agreement with the degree of decidualization.

In early pregnancy, decidualization was not uniform over the cells of the decidua, in which case the better decidualized cells indicated by the bigger cell size had the stronger immuno- and in situ staining of PRL and its mRNA. From early to mid pregnancy, decidual cells continued to increase in size and decidualization has spread over the decidua. Correspondingly, the marked increment in PRL content and its mRNA level was found in the individual cells which was consistent over the specimen of decidualized endometrium examined. Additionally,

compared with the early pregnancy, the proportion of positive in situ cells in sections increased dramatically in mid pregnancy. Furthermore, the close correlation between decidual cell size and grain density in each cell further supports the hypothesis that PRL gene transcription was switched on by decidualization in vivo, which continued throughout pregnancy. Nevertheless the present findings could not distinguish at this stage whether the factors modulating decidualization have an indirect or direct effect on activation of PRL gene expression in decidua during pregnancy.

In contrast, the decidual PRL content and its mRNA level in individual cells from mid to term pregnancy showed no obvious increase according to the overview of immuno- and in situ staining on the sections and grain density obtained by the quantitative analysis of in situ hybridization. In addition, the numbers of positive immuno- and in situ staining cells from mid to term pregnancy were constant as well, but the decidual cells continued to increase in size. The present results at this stage can not disclose the mechanism by which continuance of decidualization is not accompanied by a further increment of PRL production by term decidua. However, PRL gene expression in individual term decidual cell still paralleled the degree of decidualization, since the bigger decidual cells contained the more PRL mRNA signals, shown by in situ hybridization, and there was a close correlation between the decidual cell size and PRL mRNA level (Fig. 3.2.C).

The changes of PRL in amniotic fluid found in the present study are in consistent with those of the previous studies (McNeilly et al., 1977; Kletzky et al., 1985). There was a minor increase in PRL concentration in early pregnancy. During mid pregnancy the concentration of PRL increased dramatically to reach its maximum around 16-20 weeks of pregnancy, after which PRL concentration gradually decreased until delivery. However little information is available to explain the mechanism of the PRL pattern in amniotic fluid which will give a clue to understand the control of PRL synthesis. Thus the PRL concentrations in amniotic fluid during early, mid and term pregnancy were reexamined, in combination with the investigation of de novo

synthesis of PRL in decidua. By consideration of the volume increment of amniotic fluid (Ganong, 1984) and the net increasing rate of PRL synthesis shown by the immunocytochemistry and in situ hybridization, it can be concluded from the present results that the large increase in concentration of PRL in amniotic fluid from early to mid pregnancy must be from active synthesis by decidual cells to overcome the increment in the volume of amniotic fluid. From mid to term pregnancy, PRL production is still constant, since the numbers of positive immuno- and in situ staining cells and intensity of immuno- and in situ staining within these cells were maintained as revealed by immunocytochemistry and in situ hybridization. Hence, the decreasing PRL concentration measured in amniotic fluid probably resulted from the dilution by the increasing amount of amniotic fluid occurring in the third trimester pregnancy as suggested previously by McNeilly et al. (1977).

The present results clearly show that PRL production during human pregnancy corresponded to the degree of decidualization. This suggests that PRL production by the pregnant decidua is still, at least partly, under the control of the sex steroids, since it is well established that decidual cells are the main targets of the sex steroids, under which influence decidual cells undergo further decidualization. Progesterone alone failed to stimulate PRL production by pregnant decidua as discussed above indicating that the decidualization process might be more complex during pregnancy. Whether both oestrogen and progesterone are required for maintaining the decidualization and consequent PRL production or other factors derived from placenta (Handwerger et al., 1987a) augment the decidualization initiated by the sex steroid hormones need to be further investigated.

Conclusions: this is the first study to examine decidual PRL regulatory mechanism at the level of both synthesis and secretion, by combining in situ and Northern hybridization to estimate gene expression with the immunocytochemistry to qualify intracellular PRL synthesis and measurement of secreted PRL by radioimmunoassay of PRL in amniotic fluid. The present striking finding was that there was a close correlation between PRL gene expression and decidualization in vivo.

Further studies will be carried out to examine the effect of the factors involved in decidualization on the control of decidual PRL production.

Sex Steroid Regulation of Prolactin Production by Human Early and Term Decidua

Part I. The effect of exogenous progesterone, oestradiol, and progesterone in vitro on prolactin production by the human decidua in early pregnancy

4.1.1. Introduction

PRL production by endometrium from the normal menstrual cycle can be induced and stimulated by progesterone *in vivo* (Ying et al., 1988) and *in vitro* (Joly et al., 1989a). This PRL production is related to histological decidualization of endometrium (Joly and Redback, 1979). The previous chapter has shown a close relationship between decidualization, PRL production and PRL gene expression throughout pregnancy. Since progesterone alone had little effect on PRL production of pregnant decidual as demonstrated by earlier studies using *in vitro* culture (Joly and Redback, 1984, 1985 and Brown et al., 1989), it is of interest how important the effects of progesterone on PRL production are during early pregnancy. The study in Part I of this chapter was designed to investigate the effects of blocking the action of progesterone with the anti-progestin mifepristone (RU-486) *in vivo* on PRL production in decidua parietalis, free of trophoblast, and decidua capsularis, with attached trophoblast.

4.1.2. Materials and methods

This study was approved by the Paediatric/Reproductive Medicine Ethics of Medical Research Committee of the London Health Board and the patients gave consent to the study and collection of tissue was undertaken by Dr. J. Wilson, and Dr. D. T. Baird, Department of Obstetrics and Gynaecology, University of Edinburgh. All patients were referred to the Department of Obstetrics and Gynaecology, Simpson Maternity Hospital (S.M.H.) of the Royal Edinburgh Infirmary.

Sex Steroid Modulation of Prolactin Production by Human Early and Term Decidua

Part I. The effects of the anti progestin, mifepristone, in vivo, and progesterone in vitro on prolactin production by the human decidua in early pregnancy

4.1.1. Introduction

PRL production by endometrium from the normal menstrual cycle can be induced and stimulated by progesterone in vivo (Ying et al., 1988) and in vitro (Daly et al., 1983a). This PRL production is related to histological decidualization of endometrium (Maslar and Riddick, 1979). The previous chapter has shown the close relationship between decidualization, PRL production and PRL gene expression throughout pregnancy. Since progesterone alone had little effect on PRL production of pregnant decidua as demonstrated by earlier studies using in vitro culture (Rosenberg and Bhatnagar, 1984; Ren and Brausterin, 1990), it is not clear how important the effects of progesterone on PRL production are during early pregnancy. The study in Part I of this Chapter was designed to investigate the effects of blocking the action of progesterone with the anti progestin mifepristone (RU 486) in vivo on PRL production in decidua parietalis, free of trophoblast, and decidua capsularis, with attached trophoblast.

4.1.2. Materials and methods

This study was approved by the Paediatric/Reproductive Medicine Ethics of Medical Research Subcommittee of the Lothian Health Board and the clinical management of the patients and collection of tissue was undertaken by Dr. J. Norman and Professor D. T. Baird, Department of Obstetrics and Gynaecology, University of Edinburgh. All patients gave informed consent and were treated in ward 54 of Simpson Maternity Pavilion (SMMP) of the Royal Infirmary, Edinburgh.

4.1.2.1. Tissues collection

Human decidua from early pregnancy (42-56 days), calculated from the last menstrual period, was obtained by curettage during therapeutic abortions. Decidua was obtained from 7 women who had been treated with a single oral dose of 600 mg of mifepristone (kindly supplied by Dr D. Philibert, Roussel Uclaf, Paris, France) 24 to 36 h before tissue collection and from a further 8 women without any treatment in vivo as the control group. At the time of surgery, the decidual tissue was immediately placed in a sterile bottle that contained RPMI 1640 Medium (Gibco Limited, Paisley, Scotland, UK) supplemented with 200 µg/ml gentamycin (Sigma Chemical Co., St. Louis, MO, U.S.A.), 2 µg/ml amphotericin B (Sigma) for transportation.

4.1.2.2. Development of the organ culture method

The organ culture was chosen for this study based on the following major advantages:

1. Maintenance of histological and biochemical integrity which was essential for investigating the tissue received in vivo treatment;
2. Preservation of cell and matrix interaction, especially when the cell interaction between trophoblast and decidual cells needed to be preserved to mimic the in vivo status in decidua capsularis;
3. Ease of performance.

Several parameters i.e. selection of medium, gas phase, substrates, and sterilization were investigated to ensure that the organ culture system was satisfactory for this study.

As the choice of medium is empirical, RPMI-1640 medium (Gibco) was selected for this study since this was used in the previous studies on organ culture carried out in the laboratory of MRC Reproductive Biology Unit, Edinburgh, UK (Dr R. Kelly). For better tissue maintenance in vitro, 5% (v/v) calf serum (Gibco) previously stripped with 1 mg/ml charcoal was added to the culture medium.

The equipment involved in dissection procedure was autoclaved and the

stripped calf serum were sterilized by passing through a 0.2 μm filter. Furthermore, all dissection was performed in a laminar-flow hood.

Initially the decidua was incubated on a piece of sponge (12x10x10 mm³) sterilized with 10% Shield surrounded by 1 ml of culture medium, so that the tissue was placed at the inter-phase of air and medium to prompt the oxygen to penetrate into the tissue. However, tissue death during culture was noted on several occasions. This was related to trace amount of the detergent used for sterilization which could not be completely removed from the sponge. Thus, in subsequent experiments the sponge was not utilized and the tissue was incubated in suspension culture. The tissue was cultured in 24 well plates under 95% air with 5% carbon dioxide.

Sterile 24-well disposable plastics plates (Nunc, Nunc, Denmark) treated to give a net negative charge were used throughout the organ culture. As the medium in this study was changed daily, the amount of tissue in each well was optimized (4 pieces per well; approximately 2 mm³ each) to prevent the fall of pH to below 7.0 which would indicate a shortage of nutrients.

The tissue viability was examined histologically on cryostat sections (5 μm) after 3 or 6 days in culture in three separate tissues. After in vitro culture for 3 days, the integrity of cell and tissue architecture was maintained. However, after 6 days culture, some central necrosis could be identified in the tissue. Thus, all the studies were terminated on day 5.

The final procedure for the organ culture was as follows:

The medium, RPMI 1640, used for long term organ cultures was supplemented with previously stripped 5% (v/v) calf serum, 50 $\mu\text{g}/\text{ml}$ gentamycin and 2 $\mu\text{g}/\text{ml}$ amphotericin B. The final pH of complete medium was 7.3 to 7.4. All dissection was performed in a laminar flow hood where the tissue was rinsed twice in fresh medium, divided into decidua parietalis which was free of fetal tissues and decidua capsularis in which trophoblast cells were attached to the decidua. The

tissues were then minced into small pieces approximately 2 mm³ and four pieces of decidua parietalis or capsularis (each weighing about 2 mg) were selected and placed in each well of 24 well plates and 1 ml of culture medium was added to all wells. Control cultures received complete RPMI 1640 medium only. Test cultures containing decidua parietalis received medium containing 0, 50 or 500 ng/ml progesterone, or 50 ng/ml progesterone and 175 ng/ml mifepristone. Decidua capsularis received either complete RPMI 1640 medium or 500 ng/ml progesterone. Tissue cultures from the group which received the mifepristone in vivo were treated in exactly the same way as the control group. There were 4 replicates for each treatment. The plates were maintained at 37°C in an incubator gassed with 95% air and 5% CO₂. The culture medium was changed daily. The spent medium was frozen and stored at -20°C prior to assay for human PRL.

4.1.2.3. Radioimmunometric assay of decidual prolactin

Samples of culture medium were assayed for decidual PRL using a 2-site immunoradiometric assay (IRMA) for human PRL (NETRIA, St Bartholomews Hospital, London, U.K.) as described in chapter 3.

4.1.2.4. Avidin-biotin immunoperoxidase staining

At the time of tissue collection, a portion of each tissue was fixed in 4% (w/v) buffered formalin, pH 7.4, for histological examination and avidin-biotin immunoperoxidase staining. After embedding in paraffin wax, sections (3µm) were cut and stained with hematoxylin and eosin (HE) or immunostained for PRL by avidin-biotin immunoperoxidase staining methods using rabbit anti-human PRL serum (Lot No. 068, DAKO Corporation, Santa Barbara, CA, U.S.A.). The cells size in HE sections was measured by an Imagan 2 programme. The immunostaining was performed as described in Chapter 3.

4.1.2.4. Analysis of Data

The amount of PRL accumulated in the medium removed from cultures that were prepared from the same sample of decidua and received the same treatment in vitro were averaged to provide a single representative value for an individual patient ($n=1$). These means were then combined to provide an overall mean \pm s.e.m. for each day of culture in the various treatment groups. The data were then analysed by paired Student's t test for the same treatment on different days. The effect of different treatments on the same day was analysed using unpaired Student's t -test, which was also used to analyse the effect of mifepristone on cell size from decidua parietalis and capsularis.

4.1.3 Results

4.1.3.1. Pattern of PRL production by decidua

A. Control

As shown in Fig 4.1. PRL production by decidua parietalis during the five days of organ culture in vitro in the absence of progesterone decreased significantly ($P<0.05$) on each day. Addition of 50 and 500 ng/ml progesterone in vitro slightly increased decidual PRL production compared with control tissue receiving medium alone but this was not significant ($P>0.05$). The addition of mifepristone with 50 ng/ml progesterone in vitro had no effect on PRL production by decidua parietalis.

In contrast, for decidua capsularis there was no significant ($P>0.05$) decrease in PRL production during the five days in organ culture, regardless of the presence or absence of progesterone (Fig. 4.2.). During the first two days in culture decidua parietalis produced more PRL than the decidua capsularis, while in the last two days decidua capsularis produced more PRL than that of decidua parietalis (Fig. 4.3.).

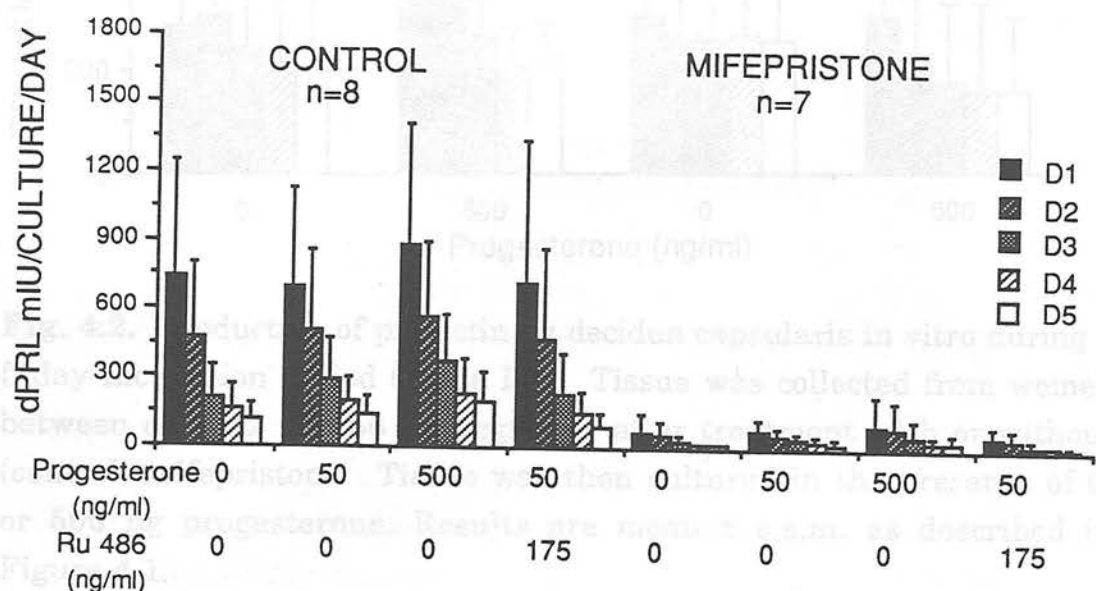


Fig.4.1. Production of PRL by decidua parietalis in vitro during a 5 day incubation period (D1 to D5). Tissue was collected from women between days 42 and 56 of pregnancy after treatment with or without (control) mifepristone (RU 486). Tissue was then cultured in the presence of 0, 50 or 500 ng progesterone or 50 ng progesterone and 175 ng mifepristone. The amounts of decidual PRL accumulated in the medium removed from cultures that were prepared from the same sample of decidua and received the same treatment in vitro (n=4 replicates per treatment) were averaged to provide a single representative value for an individual patient (n=1). These means were then combined to provide an overall mean \pm s.e.m. for each day of culture in the various treatment groups.

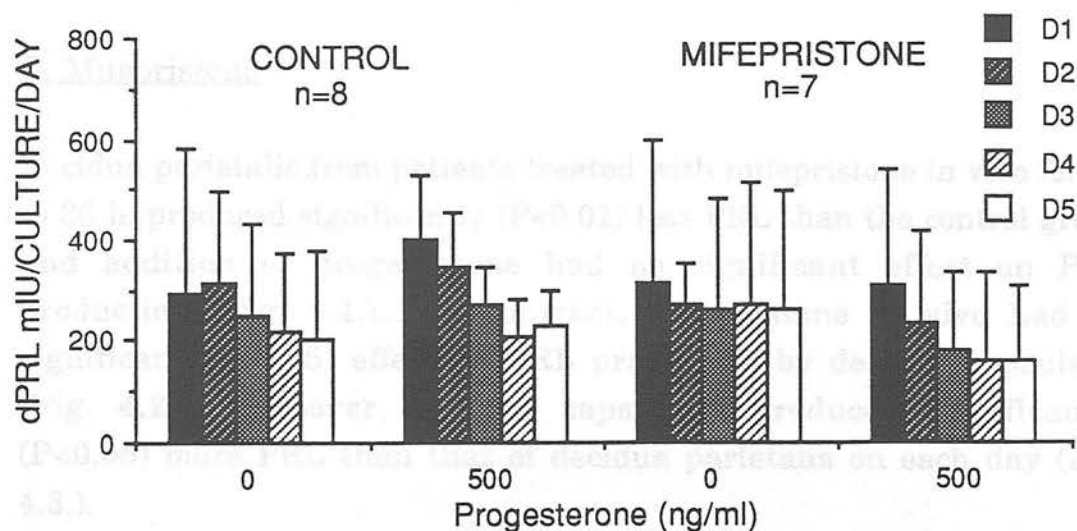


Fig. 4.2. Production of prolactin by decidua capsularis in vitro during a 5 day incubation period (D1 to D5). Tissue was collected from women between days 42 and 56 of pregnancy after treatment with or without (control) mifepristone. Tissue was then cultured in the presence of 0, or 500 ng progesterone. Results are mean \pm s.e.m. as described in Figure 4.1.

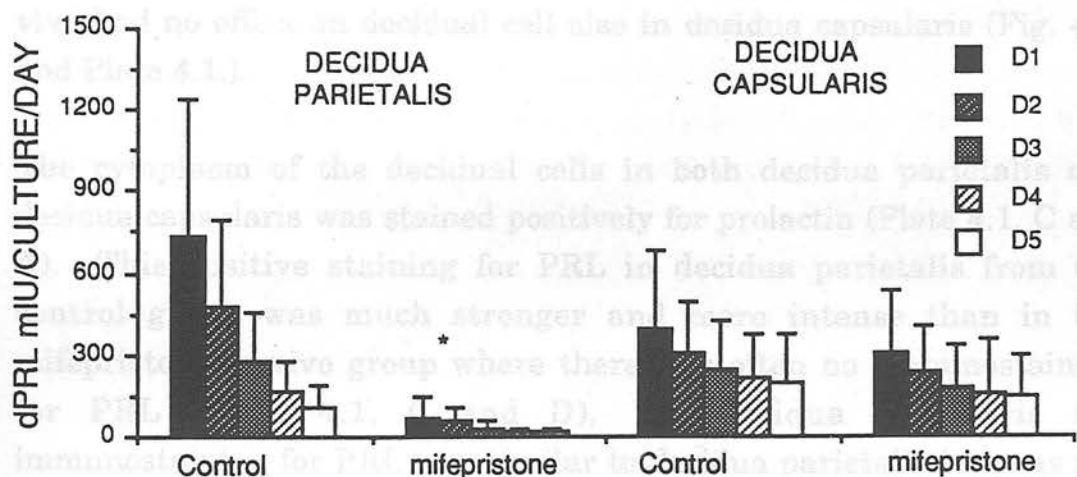


Fig. 4.3. Comparison of prolactin production by decidua parietalis and capsularis in vitro during a 5 day incubation period (D1 to D5). Tissue was collected from women between days 42 and 56 of pregnancy after treatment with or without (control) mifepristone. Results are mean \pm s.e.m. as described in Fig. 4.1.

B. Mifepristone

Decidua parietalis from patients treated with mifepristone in vivo for 24 to 36 h, produced significantly ($P < 0.01$) less PRL than the control group and addition of progesterone had no significant effect on PRL production (Fig. 4.1.). In contrast, mifepristone in vivo had no significant ($P > 0.05$) effect on PRL production by decidua capsularis (Fig. 4.2.). Moreover, decidua capsularis produced significantly ($P < 0.05$) more PRL than that of decidua parietalis on each day (Fig. 4.3.).

4.1.3.2. Histology and avidin-biotin immunoperoxidase staining

Decidua parietalis only contained maternal tissue with typical decidual cells located around the hypersecretory glands, and some fibroblast cells and lymphocytes were present throughout the section. Both decidual and trophoblast cells were found in decidua capsularis. There was a greater abundance of blood vessels in the decidua parietalis than decidua capsularis. Cells in decidua parietalis from the women pretreated with mifepristone in vivo were significantly ($p < 0.001$) smaller when compared with the control group, while mifepristone in vivo had no effect on decidual cell size in decidua capsularis (Fig. 4.4. and Plate 4.1.).

The cytoplasm of the decidual cells in both decidua parietalis and decidua capsularis was stained positively for prolactin (Plate 4.1. C and A). This positive staining for PRL in decidua parietalis from the control group was much stronger and more intense than in the mifepristone in vivo group where there was often no immunostaining for PRL (Plate 4.1. C and D). In decidua capsularis the immunostaining for PRL was similar to decidua parietalis but was not affected by pretreatment in vivo with mifepristone (Plate 4.1. A and B).

Glands were only observed in the decidua parietalis in the sections used for immunostaining. While these glands showed intense staining for PRL in the controls, this was greatly reduced in the mifepristone in vivo group.

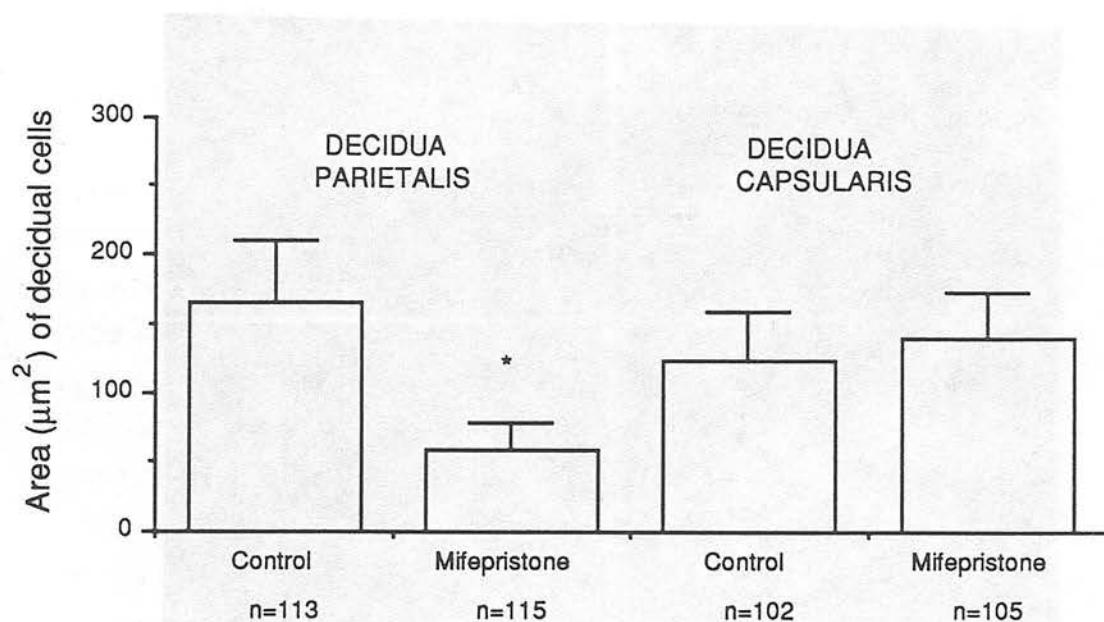


Fig. 4.4. Comparison of the area of decidual cells in sections (HE stain) of decidua parietalis and decidua capsularis taken from women treated with or without (control) mifepristone. Results are mean \pm s.e.m. n = no. of cells measured per treatment. * $p < 0.001$ compared with control.

4.1.4 Discussion

The present study shows that PRL is expressed in decidual cells *in vivo* in the human endometrium. The morphology and number of decidual cells are similar to those

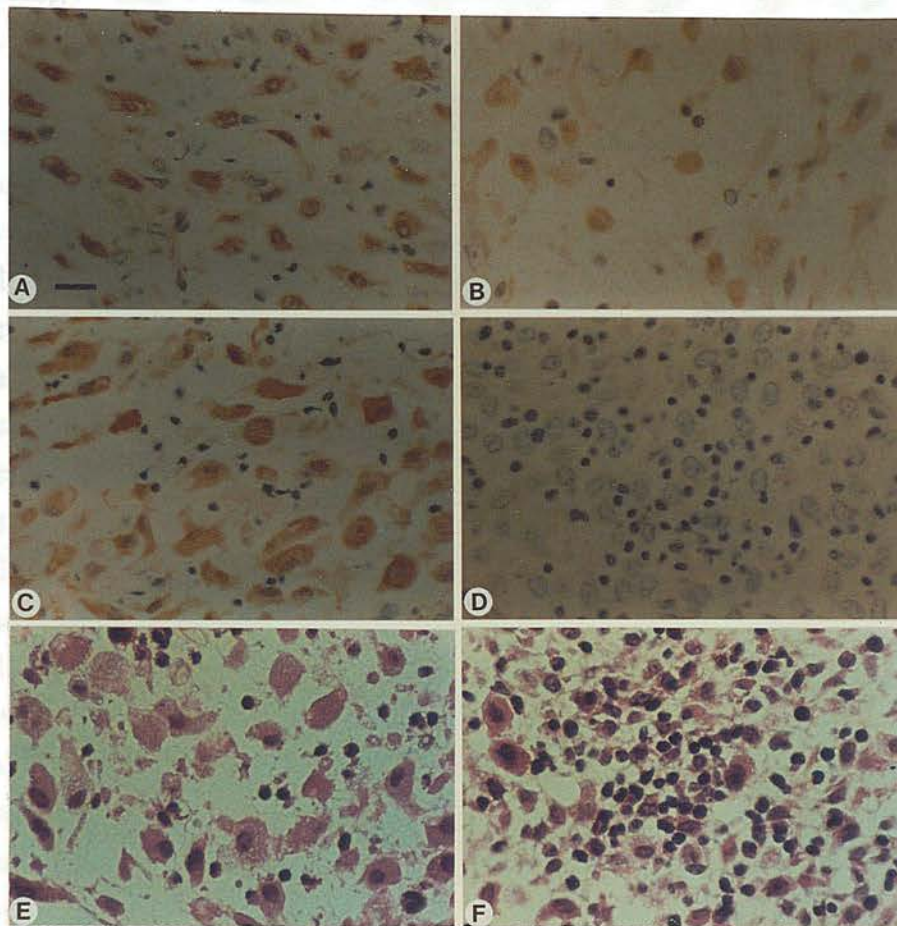


Plate 4.1. Avidin-biotin immunostaining of PRL and HE stain in sections of decidua parietalis (c,d), (e,f-HE stain) and decidua capsularis (a,b) from women treated without (a,c,e) or with (b,d,f) mifepristone for 24 to 36 h before collection of tissue. Note that pretreatment with mifepristone resulted in complete absence of PRL staining only in decidual cells of decidua parietalis and the presence of pycnotic nuclei in these cells.

4.1.4 Discussion

The present results suggest that PRL production by decidua parietalis in vivo in early pregnancy was dependent on progesterone. The morphology and results of immunostaining for PRL suggest that blockade of progesterone action in vivo results in a reduction in decidual cell size, PRL cell content and the degree of decidualization. These results support the concept that PRL production by decidual cells occurred as a consequence of decidualization which was induced by progesterone (as described in Chapter 3). In addition these results extend previous study in vitro of effect of progesterone on PRL production from early pregnancy (Maslar et al., 1988). In spite of the apparent dependence of PRL secretion on progesterone in vivo, it is of interest that addition of progesterone in vitro could not stimulate decidual PRL secretion, confirming previous reports on decidual PRL production by decidua in vitro from early human pregnancies (Bischof et al., 1986; Ren and Braustein, 1990). It has been observed that decidua capsularis released between 3 and 5 fold more progesterone than decidua parietalis during the incubation period (Chapter 5), but the concentration of progesterone was 10 to 90 times less than the maximum amount of progesterone added in vitro. Thus it is unlikely that endogenous production of progesterone could explain the difference in the release of PRL between decidua capsularis and parietalis. Additionally, It is well known that oestrogen priming is necessary for demonstration of a progestational effect (Tamaya et al. 1985; Irwin et al., 1989). This may explain the absence of an effect of progesterone on decidual PRL production, since oestradiol was not added to the culture medium.

The observations in early pregnancy are the first where mifepristone has been given in vivo but the results complement previous in vitro studies which showed that addition of mifepristone to decidualized tissues from the late luteal phase and early pregnancy prevented progesterone induction of decidual PRL production (Bischof et al., 1986; Huang et al., 1987). In the present study addition of mifepristone to the cultures in vitro, in the presence of progesterone, did not affect decidual

PRL production. It is possible that this local effect of mifepristone is due to an inadequate concentration to displace all the progesterone bound to receptors and will need further investigation.

It is well documented that during the luteal phase of the human menstrual cycle, the onset of prolactin secretion by proliferating endometrium in culture corresponded to the onset of histologically apparent decidualization in response to progesterone-induced differentiation in vitro (Daly et al., 1983a). Similarly, decidualized endometrium from non-pregnant women in vitro maintained prolactin secretion and histological integrity only in the continued presence of progesterone (Maslar et al., 1986). However, little information is available to elucidate the relationship between decidualization and progesterone on PRL production during early human pregnancy. The present results are the first from biological and morphological features where the inhibition of PRL production resulted from decidualization of decidual parietalis by mifepristone in vivo, which indicated an indirect effect of progesterone on regulation PRL production during pregnancy.

Of considerable interest is the observation in the present study that the secretion of PRL by decidual cells of the decidua capsularis did not decline over the 5 days in culture period, in contrast to the decidua parietalis. In addition, mifepristone in vivo did not affect PRL production by decidua capsularis. These results suggest that the trophoblast tissue attached to the capsularis may produce a factor(s), steroids or non-steroids, which maintained decidualization and, hence, decidual PRL production, in spite of progesterone withdrawal. There was evidence to imply that decidua parietalis and capsularis were under progesterone control, because progesterone receptors have been localized in both decidual parietalis and capsularis (Chapter 5). It was also possible that the greater abundance of blood vessels in the decidua parietalis than decidua capsularis increased the exposure of decidual cells of the parietalis to mifepristone.

On the other hand, potential PRL releasing factors have been identified in the utero-placental unit. These factors include insulin (Thraikill et

al., 1989), insulin-like growth factor I (IGF-1) (Thraikill et al., 1988), human chorionic gonadotrophin (hCG) (Rosenberg and Bhatnagar, 1984) and a placental-derived PRL releasing factor (Handwerger et al., 1987a). Moreover, it has been observed that significantly higher concentrations of PRL were associated with these tissues in which definitive villi containing cyto- and syncytio- trophoblast cells were identified more than 2 weeks post conception, compared to those less than 2 weeks post conception in which the syncytial trophoblast had not yet differentiated extensively (Maslar et al., 1980). These results indicate that progesterone plays a central role in switching on PRL production as a result of decidualization of the endometrium. Apart from progesterone, a factor(s) produced by fetal tissues (trophoblast cells), steroids or non-steroids may be required for maintaining decidualization and PRL production throughout pregnancy.

Summary: the present results show for the first time that blockade of progesterone action in vivo by the anti progestin mifepristone in early human pregnancy results in a suppression of decidual PRL production associated with morphological de-decidualization of decidual cells in the decidua parietalis. In contrast, withdrawal of progesterone did not affect either decidualization or PRL production by decidual cells of the decidua capsularis. These results suggest a local control of PRL derived from the trophoblast is associated with stimulating and maintaining PRL production during pregnancy.

Part II. The combined effect of progesterone and oestrogen on prolactin production by human term decidual cells

4.2.1. Introduction

Prolactin production by decidua capsularis to which trophoblast cells attached was sustained for up to 5 day in in vitro culture with morphological maintenance of decidualization even under the condition of progesterone withdrawal in vivo. This suggests that a factor(s) from the trophoblast prevent de-decidualization even when progesterone action is blocked by anti progesterone. Furthermore, PRL production was correlated with decidualization in either normal menstrual cycle (Daly et al., 1981) or pregnancy (Chapter 3). It is generally accepted that progesterone is required for the induction of decidualization and the role of progesterone is constant throughout the lifespan of decidualized endometrium, i.e. that of the stimulation and maintenance of the decidual reaction. In contrast, the role of oestrogen is less defined and has not yet been clearly elucidated. The previous results in Part I showed that progesterone alone could not stimulate PRL production, while a factor(s) derived from trophoblast could maintain decidualization, and thus maintain PRL production. During pregnancy, large quantities of progesterone and oestrogen are produced by syncytio-trophoblast cells. Presumably progesterone receptors in decidua depend on oestrogen stimulation, hence, there may be an indirect association between oestrogen and decidualization (Tamaya et al., 1985), which is in turn related to PRL production. To determine whether both steroids are required for the continued release of PRL, the pattern of PRL production produced by the cultured term decidual cells in vitro treated with or without progesterone and oestrogen, either alone or in combination, was investigated in this part of study.

While organ culture has been employed in some studies in this thesis, the present studies were undertaken using cell culture. An in vitro cell culture system was chosen because, firstly, it is possible to select the cell population for study using cell separation techniques; secondly, tissue viability appears better during in vitro term cell culture; finally, the system showed less variation in response.

4.2.2. Materials and methods

4.2.2.1. The development of cell culture method

Because of the limited availability of tissue from other stage of pregnancy, only term decidua was used in these studies. Several aspects of the preparation of the term decidual cells and the subsequent culture conditions were investigated before the methods was utilized, which include:

1. Enzyme treatment for cell dispersion;
2. Plating efficiency on uncoated or collagen-coated culture plates;
3. Semi-purification of decidual cells.

A. Enzymatic digestion of tissue and culture

The term placenta with membranes from uncomplicated pregnancies between 37-40 week of gestation were received in the labour ward immediately after delivery. When the decidua was separated using a scalpel, the areas with obvious attachment of trophoblast were avoided. The separated decidua was collected into PBS supplemented with 50 µg/ml gentamycin and 5 µg/ml amphotericin B and then transported to the laboratory for further processing.

Decidual cells were dispersed according to the method of Markoff et al. (1983a) with some modification as described below:

Because collagen and hyaluronic acid are the natural substrates upon which decidual cells migrate in vivo, collagenase and hyaluronidase were chosen for digesting decidual tissue. Two different types of collagenase (type III, Lot no. C-0255; type I, Lot no. C-0130; Sigma) were compared for the yield and viability of the dispersed cells. The yield of the dispersed decidual cells obtained from two different collagenases was similar, but the viability of the dispersed decidual cells using collagenase III was often less than 50%. Thus the collagenase I was selected in the subsequent experiments carried out in this study.

However, individual batches of collagenase, even of same type and from the same source, varied greatly in their activity and ability to digest decidual tissue is in agreement with other workers (Findlay et al., 1990). Therefore, optimum digestion times, were tested in order to choose the most suitable preparation for producing an acceptable decidual cell yield and cell viability. In a previous study (Markoff et al., 1983a), 0.1% (w/v) collagenase of 120-150 U digesting activity/mg and 0.1% (w/v) hyaluronidase with a digesting activity of 255 U/mg were used. Thus in the present study, in which different preparation with different digesting activity of the enzymes were employed, the percentage of both collagenase and hyaluronidase were varied to maintain a similar unitage of activity. When the digesting activity of collagenase purchased was at 120-150 U/mg, 0.1% (w/v) collagenase was used in the culture system. If the collagenase digestion activity was higher than 150 U/mg, the concentration of collagenase used for dispersing decidual cells was decreased correspondingly, which was the essential step to obtain the better cell viability in this cell culture system. The concentration of 0.1% (w/v) hyaluronidase (type I-S, Lot no. H-3506, Sigma) was used in this study with the digesting activity around 200-250 U/mg.

After the concentrations of the enzymes were decided, different digestion times, i.e. 1 h, 1.5 h and 2 h, were compared to obtain the best cell viability and cell yield.

Table 4.1.

Time	Cell yield (cells/g. tissue)	Cell viability
1 h	1.5×10^6	>98%
1.5 h	4×10^6	80-90%
2 h	$4.5-5 \times 10^6$	60-80%

To compromise between cell viability and cell yield, all the enzymatic digestion of decidua was carried out for 1.5 h at 37°C with the gentle stirring. It was essential in this culture system to disperse cells by trituration through a 50 ml eppendorf, otherwise only a few decidual

cells could be collected from the enzyme solution.

B. The plating efficiency using uncoated or collagen coated plates

In order to increase the plating efficiency of the cells, multiwell plates were coated with collagen (type I, Sigma) which is the main component of the extracellular matrix in decidua (see **Appendix 7** for the detailed coating method). However, no significant difference in PRL production between plates coated with or without collagen was found under this culture conditions. Thus uncoated plates were used during the subsequent culture procedure.

C. Cell separation using Percoll

When the same density of cells was plated into wells, the yield of PRL produced by cells with 40% Percoll purification (the detailed method for preparing 40% Percoll shown in **Appendix 8**) was almost twice as much (1245 ± 279 s.e.m. mU/L) as that without applying Percoll purification (741 ± 83 s.e.m. mU/L). So 40% Percoll purification was the ordinary procedure undertaken in this culture procedure. After centrifugation of the dispersed cell suspension through 40% Percoll gradients, decidual cells were banded at the density between 1.019-1.033 g/ml calibrated by Density Marker Beams, which was in accordance with the previous report (Markoff et al., 1983a).

D. Immunocytochemistry and in situ hybridization

The proportion of PRL producing cells after Percoll separation, was determined using both immunostaining for PRL using anti-human PRL serum, and localization of PRL mRNA using human PRL cDNA probe by in situ hybridization. Dispersed cells were plated in tissue culture chamber slides (Nunc) at a density of 5×10^4 cells/ml in 5 ml RPMI-1640 medium supplemented with 10% fetal calf serum, 50 µg/ml gentamycin, 25 U/ml penicillin, 25 µg/ml streptomycin, and 5 µg/ml amphotericin B. The cells were then incubated for 48 h at 37°C in a humidified atmosphere of 95% air:5% CO₂ to allow attachment of the

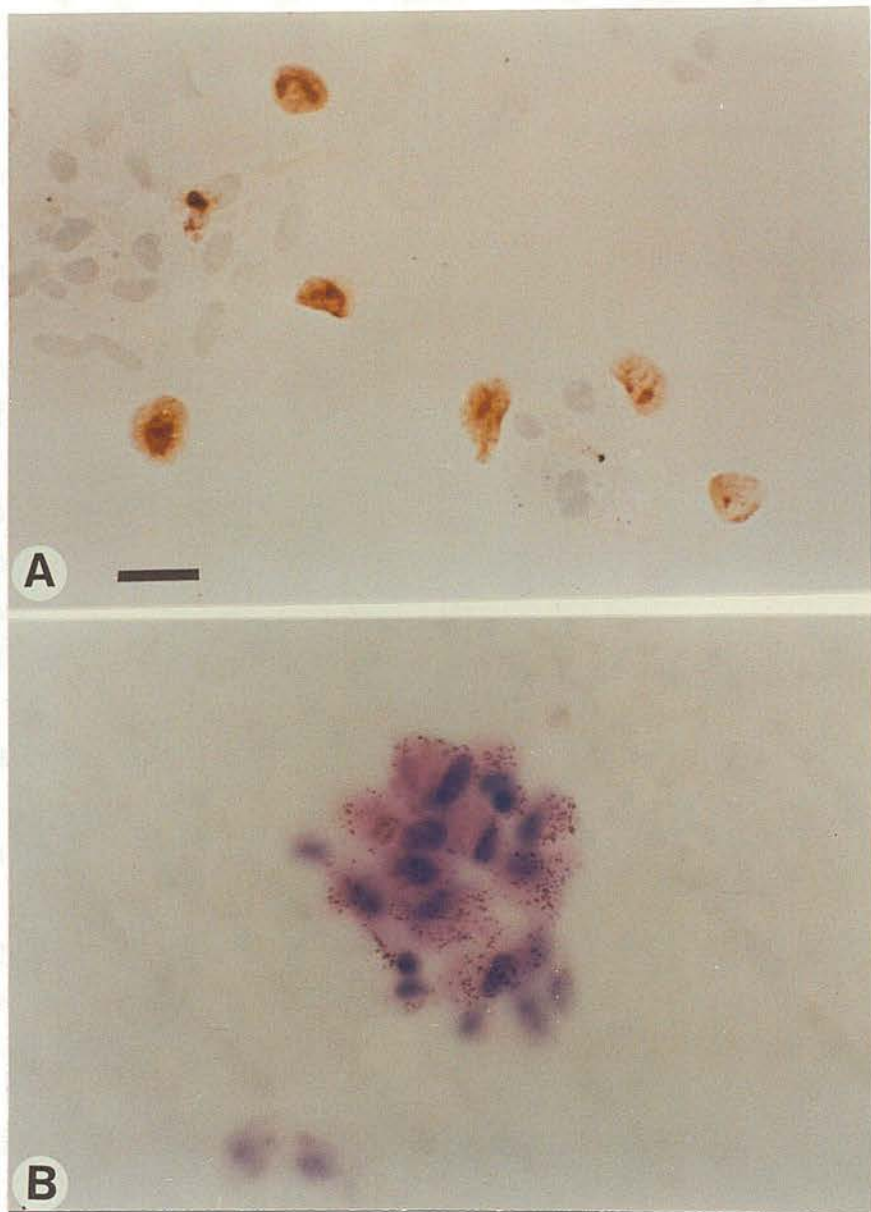


Plate 4.2. Immunocytochemical staining of PRL and in situ localization of PRL mRNA in dispersed decidual cells from human term decidua.

- (4.1.A) The specific immunostaining of PRL in dispersed term decidual cells grown in culture for 48 h.
- (4.1.B) In situ localization of PRL mRNA in dispersed term decidual cells grown in culture for 48 h. Note highly specific localization of black grains within the cytoplasm of decidual cells.

cells to the slides. The culture medium was then aspirated from the chambers, the cells were washed twice with RPMI-1640 medium and the slides were stored at -70°C until processing for immunocytochemistry or in situ hybridization.

The immunocytochemistry and in situ hybridization were performed as described in Chapter 2 except that cells were only fixed in 4% paraformaldehyde in PBS for 10 min followed by two washes in PBS for 10 min before adding prehybridization buffer or normal swine serum as blocking reagent for in situ hybridization and immunocytochemistry. Thereafter the methods were identical to those described in Chapter 2.

When the positive immuno- and in situ staining cells were counted throughout the slides, 50-60% cells were positively immunostaining with anti-PRL antibody, and 80% cells contained PRL mRNA localized by in situ hybridization (Plate 4.2. A and B).

The final protocol of decidual cell culture was as follows:

Decidual tissue from term pregnancies stripped from the chorion was washed thoroughly in PBS (Gibco) supplemented with 50 $\mu\text{g}/\text{ml}$ gentamycin and 5 $\mu\text{g}/\text{ml}$ amphotericin B. The tissue was then minced into small pieces (5 mm^3) and placed in a stirrer flask (1 g tissue/5 ml medium) and digested for 1.5 h at 37°C with gentle stirring in buffered RPMI-1640 medium containing 0.1% collagenase (type I, 120-150 units/ml, Sigma), 0.1% hyaluronidase (type I-S, 200-250 units/mg, Sigma), and 1% fetal calf serum (Flow Laboratories, Rickmansworth, Herts, UK). Cells were then dispersed by trituration through a 50 ml eppendorf sterile combitip, passed through nylon cloth (Lockertex, Warrington, Cheshire, UK; mesh size 150 μm) and then collected by centrifugation (100 x g, for 10 min). The cell pellet was washed twice and resuspended in the medium at a density $6-8 \times 10^6$ cells/ml.

One ml of dispersed cells was carefully layered on the top of the performed 40% Percoll (Pharmacia Ltd, Milton Keynes, Bucks, UK) gradients and cells were separated by centrifugation at 800 g for 20 min at room temperature. Those with densities of 1.019-1.033, calibrated

with Density Marker Beans (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden), were accumulated by aspiration, and washed twice in medium. Cells were counted and assessed for viability by trypan blue exclusion. Only those cells with viability more than 80% were plated in 24-well plates (Nunc) at a density of $0.5-1 \times 10^6/\text{ml}$ in 1 ml RPMI-1640 medium supplemented with 10% stripped fetal calf serum, 50 $\mu\text{g}/\text{ml}$ gentamycin, 25 U/ml penicillin, 25 $\mu\text{g}/\text{ml}$ streptomycin, and 5 $\mu\text{g}/\text{ml}$ amphotericin B. and treated as experimental design.

4.2.2.2. Steroid hormone treatment

After plating, the cells were incubated for 48 h at 37°C in a humidified atmosphere of 95% air:5% CO₂ to allow attachment of the cells to the wells. The culture medium was aspirated from the wells, cells were then treated as experimental design, with medium changed daily for 3 days and frozen for later assay of PRL.

In order to add oestradiol and progesterone to the medium, concentrated stock solution were firstly prepared by dissolving 2 mg of each steroid in 2 ml of absolute ethanol. These stock solutions were further diluted in RPMI-1640 medium to the desired working concentrations.

Nine different steroid treatments were investigated in this study, with triplicates for each treatment. This was repeated using 3 separate tissues. Control cultures received RPMI-1640 culture medium only, but with same concentration of ethanol as test cultures and test cultures received RPMI-1640 medium containing 1 or 100 ng/ml of progesterone or 1 or 10 ng/ml of oestradiol, either alone or in combination as shown in Fig 4.5. The steroids were added to the culture medium when cells were being added to the plates at the start of the culture.

4.2.2.3. Radioimmunoassay and statistical analysis

PRL radioimmunoassay was performed as described in Chapter 3. The PRL values prepared from same sample and receiving the same

treatment in vitro were averaged to provide a single representative mean for each patient. These means were then combined to provide an overall mean of different patients for each day of culture in the various treatment groups. Statistical analysis was performed by using analysis of variance and Duncan's multiple range test with significance defined as $P < 0.05$.

4.2.3. Results

The amount of PRL released by decidual cells during 3 days in in vitro culture in RPMI-1640 medium only or containing either oestradiol or progesterone or both is shown in Fig. 4.5. A-C. There was no significant effect on PRL production by decidual cells treated with either oestradiol or progesterone alone relative to non-hormonal control during in vitro culture for 3 days. However, a significant increase of PRL production occurred when 100 nM progesterone was combined with 10 nM oestradiol ($p < 0.05$) on day 1, and ($P < 0.01$) on day 2 and 3 in in vitro culture. Furthermore, on day 2 and 3, the cultured cells treated with 100 nM progesterone and 1 nM oestradiol produced significantly ($P < 0.05$) more PRL than either non-hormonal control or single steroid treated cells. Additionally, PRL production level was maintained over 3 days in vitro culture in the presence of 100 nM progesterone with either 1 or 10 nM oestradiol. However, there was no effect of addition of 1 nM progesterone with either 1 or 10 nM oestradiol.

4.2.4. Discussion

The isopycnic centrifugation of collagenase- and hyaluronidase-dispersed decidual tissue through Percoll purification resulted in the enrichment of a cell population banded at the density between 1.019-1.033, which synthesized and released PRL during in vitro culture. This result confirmed the previous report that when cells from the individual fractions of the Percoll gradient were cultured only those cells with the density between 1.017-1.045 secreted detectable amounts of PRL (Markoff et al., 1983a). The results from immunocytochemistry and in situ hybridization further confirmed that the Percoll gradient indeed separated out PRL producing cells, suggesting a reliable method

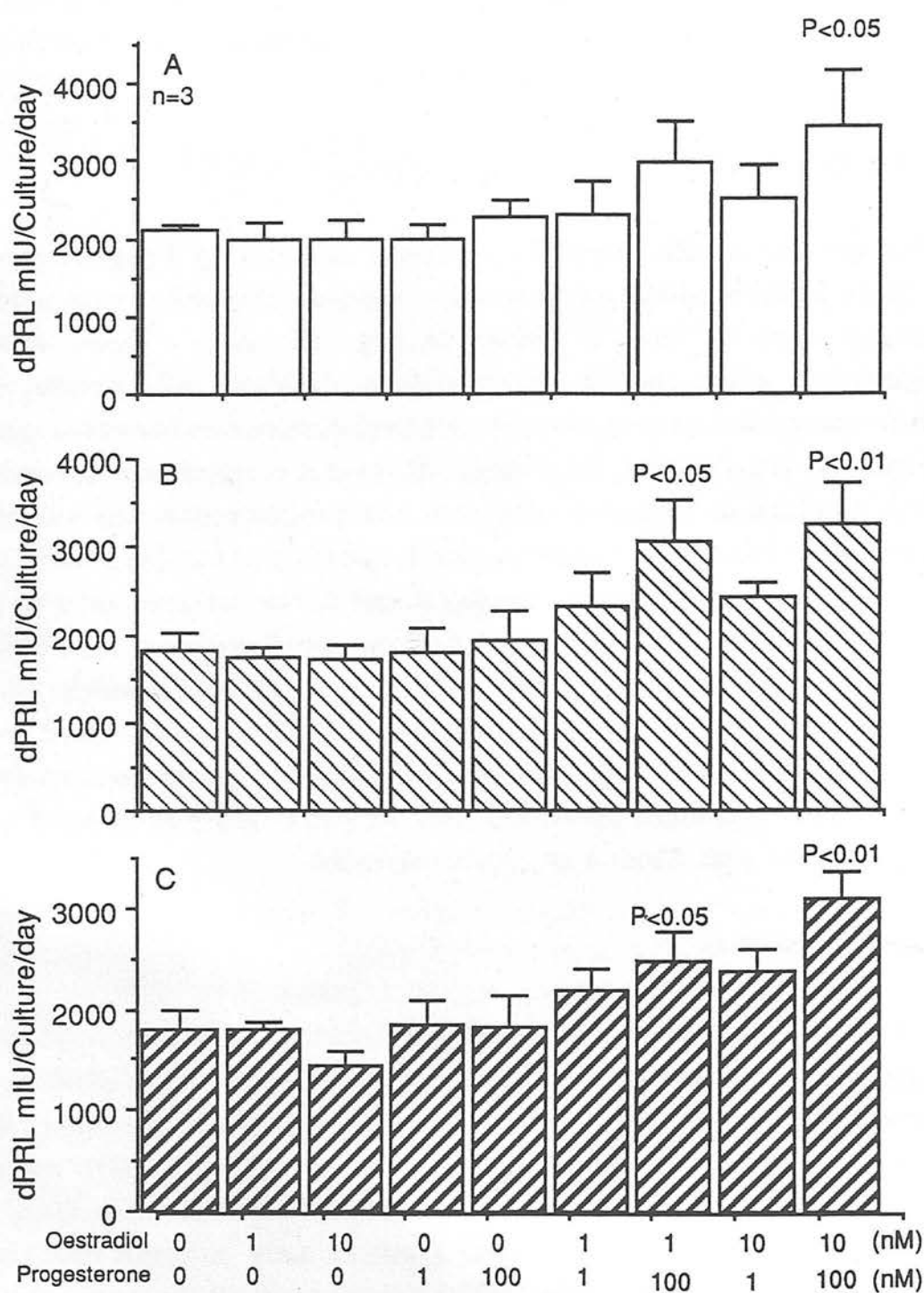


Fig. 4.5. The amount of decidual PRL released by the cultured term decidual cells in vitro during a 3 day incubation period (A-C) treated without or with 1 or 10 nM oestradiol and 1 or 100 nM progesterone, either alone or in various combination as shown in the Fig 4.5. The amounts of decidual PRL accumulated in the medium removed from cultures that were prepared from the same sample of decidua and received the same treatment in vitro (n=3 replicates per treatment) were averaged to provide a single representative value for an individual patient (n=1). These means were then combined to provide an overall mean \pm s.e.m. for each day of culture in the various treatment groups. On day 1, tissues received 10 nM oestradiol and 100 nM progesterone released significantly more ($p<0.05$) PRL than the control group or the groups received either oestradiol or progesterone alone. On day 2 and 3, tissue received 1 nM oestradiol and 100 nM progesterone or 10 nM oestradiol and 100 nM progesterone released significantly more ($p<0.05$, $p<0.01$) PRL than the control group or the groups received either oestradiol or progesterone alone.

of dispersing and separating PRL producing cells from term decidua used in this study. Much more consistent results (lower standard deviation) were obtained in the cell culture system than those of organ culture (Chapter 4, Part I).

The results presented here demonstrated that PRL production could be stimulated and maintained by the treatment of a combination of oestradiol and progesterone. The role of progesterone was defined as an initiator and stimulator of decidualization and, in turn, this had effect on PRL production in normal menstrual cycle (Maslar et al., 1986; Maslar and Ansbacher, 1988; Casslen et al., 1990). However, the mechanism of maintaining decidualization and of steroid action on PRL production during pregnancy remains unclear. A single study (Daly et al., 1983b) demonstrated that progesterone alone stimulated PRL production by term decidua, and suggested that progesterone acted as stimulator for PRL production throughout pregnancy. However, the present results in either early (Part I) or term pregnancy (Part II) showed that progesterone alone in vitro was incapable of stimulating and maintaining PRL production, in agreement with other studies (Markoff et al., 1983a; Rosenberg and Bhatnagar, 1984; Ren and Braunstein, 1990). The lack of effect of progesterone alone on PRL production was unlikely to be due to the concentration used since the dose of progesterone used in this study covered the maximal physiological range. Progesterone certainly played an important role on PRL production during pregnancy since withdrawal of progesterone in vivo using the anti progesterone mifepristone caused decidualization of decidual cells, with a dramatic decrement of PRL production (Chapter 4, Part I). This suggests that progesterone is not a direct regulator of PRL production and further supports the concept that, through decidualization in which other factors might be required, progesterone has an indirect effect on PRL production during pregnancy.

The role of oestrogen was less clear. Previous work has suggested that depending on the dose used in culture, oestrogen alone may stimulate (Rosenberg and Bhatnagar, 1984), have no effect (Irwin et al., 1989), or inhibit slightly (Daly et al., 1983b) PRL production. When combined

with progesterone, oestradiol was shown to exert negative (Daly et al., 1983a), neutral (Huang et al., 1987), or positive (Randolph et al., 1990; Casslen et al., 1990) effects on PRL production. The cellular heterogeneity of decidua tissue and the interaction between different type of cells, especially contaminated by trophoblast may contribute, in part, to the discrepancy in the steroid action of oestrogen on PRL production among different studies.

The present study has shown that oestradiol alone could not stimulate PRL production or even slightly inhibited PRL production after 3 days in in vitro culture. When applied together with 1 nM progesterone, oestradiol still had no effect on PRL production even when the concentration of oestradiol was increased 10 fold. In contrast 100 nM progesterone combined with either 1 nM or 10 nM oestradiol acted to stimulate and maintain PRL production over 3 days of culture in vitro, which indicated that the ratio of progesterone to oestrogen might be crucial for steroid action. This result is supported by the clinical evidence that abnormal progesterone or oestradiol levels during the luteal phase, resulted in a decreased ability of the endometrium to produce PRL which in turn was linked insufficient decidualization (Daly et al., 1981; 1983a).

The enhancement of progesterone-stimulated function by concurrent oestradiol treatment observed in this study is consistent with the known up-regulation of progesterone receptors by oestrogen both in vivo (Lubbert et al., 1982) and in vitro (Eckert and Katzenellenbogen, 1981). Since PRL production depends on decidualization, the study from in vitro decidual cell culture has shown that concurrent oestrogen treatment could enhance of progesterone induced decidualization (Irwin et al., 1989). In addition, oestrogen was clearly the major stimulator of cell growth and proliferation in uterus (Pavlik and Katzenellenbogen, 1978), which is a direct prerequisite for decidualization (Bell, 1983) and oestrogen priming was important for subsequent protein secretion by decidua (Seppala et al., 1988). All of these results support the concept that oestrogen might be indirectly involved in the control of decidualization and as a consequence, PRL production by decidua.

Summary: these studies clearly showed that neither oestradiol or progesterone alone maintained PRL production and suggested that oestradiol and progesterone acted synergistically to maintain decidualization and the consequent production of PRL by decidual cells. In the next chapter, studies on the cellular distribution of oestradiol and progesterone receptor are reported in the context of decidualization and PRL production.

In the previous Chapter 4, Chapter 5 it was shown that progesterone and oestradiol acted synergistically to maintain PRL production in either early or term pregnancy (Chapter 5). It has been well documented that steroid hormones can only act after binding to their specific receptors in the target tissue (Gorski and Gannon, 1976). Decidual steroid receptors must therefore play a vital role in mediation of the responses of decidua to steroids.

The changes in receptor content and receptor distribution using biochemical binding assay have been reported during human pregnancy. The results showed a progressive decrease in progesterone and oestrogen binding to undetectable level by term (Padayatchi et al., 1987). However, because of the heterogeneity of decidual cells, it has been exceedingly difficult to accurately study and determine the relative receptor contents of individual cell types, and the intracellular distribution of receptors using biochemical assays. In addition, no information is available to define the exact receptor status of various intrauterine tissues in relation to PRL production during pregnancy.

Immunocytochemistry allows direct localization of steroid receptors within individual cells and an evaluation of the receptors within a complex association of cell types in decidua parietalis and capsularis. Thus, the following studies were carried out to determine, firstly, the status of steroid receptors in decidua parietalis and capsularis; secondly, the patterns of distribution of intracellular receptors; thirdly, the changes in receptor content throughout the pregnancy; finally, the relation between decidual steroid receptors and PRL production in both decidua parietalis and decidua capsularis by examining the effect of oestradiol and progesterone on decidual steroid receptors and PRL production and decidual immunoreactive PRL and progesterone on some decidual

Immunolocalization of Oestrogen and Progesterone Receptors in the Human Decidua in Relation to Prolactin Production

5.1. Introduction

In the previous Chapter (Chapter 4) it was shown that progesterone and oestrogen acted synergistically to maintain PRL production in either early or term pregnancy (Chapter 5). It has been well documented that steroid hormones can only act after binding to their specific receptors in the target tissue (Gorski and Gannon, 1976). Decidual steroid receptors must therefore play a vital role in mediation of the responses of decidua to steroids.

The changes in receptor content and receptor distribution using biochemical binding assay have been reported during human pregnancy. The results showed a progressive decrease in progesterone and oestrogen binding to undetectable level by term (Padayachi et al., 1987). However, because of the heterogeneity of uterine cells, it has been exceedingly difficult to accurately determine the relative receptor contents of individual cell types, and the intracellular distribution of receptors using biochemical assays. In addition, no information is available to define the exact receptor status of various intrauterine tissue in relation to PRL production during pregnancy.

Immunocytochemistry allows direct localization of steroid receptors within individual cells and an evaluation of the receptors within a complex association of cell types in decidua parietalis and capsularis. Thus, the following studies were carried out to determine, firstly, the status of steroid receptors in decidua parietalis and capsularis; secondly, the changes in the pattern of intracellular receptor distribution and receptor content throughout the pregnancy; finally, the relationship among steroids, steroid receptors and PRL production in both decidua parietalis and decidua capsularis by examining the PRL and progesterone production from decidua parietalis and capsularis and double immunostaining PRL and progesterone on same sections.

5.2 Materials and methods

5.2.1. Development of method for freezing tissue

Decidua samples from early pregnancy were obtained by curettage during therapeutic abortions and term pregnancy tissue was collected in the labour ward immediately after delivery. Both early and term tissue were frozen as describe in Method 3. In each group, tissues from 3 different patients were collected both in early and term pregnancy. In early pregnancy, both decidua capsularis and parietalis were examined for oestrogen and progesterone receptors.

Previous studies have reported a loss of steroid receptor or apparent translocation of steroid receptors during tissue preparation (West et al., 1987). Since this can be avoided by using frozen sections, all immunocytochemistry of steroid receptors was performed on frozen sections.

In order to obtain good morphology of the tissue section, different freezing methods were tried:

1. A piece of tissue no more than 4 mm³ was placed in an aluminium foil and immersed in either a dry ice or liquid nitrogen bath until frozen, then stored in liquid nitrogen before performing immunocytochemistry. Even though tissue was small in size, and quickly frozen, the formation of ice crystals which disrupted the tissue and cell structure could not be prevented. This yielded heavy background staining and caused difficulty in detecting the specific staining (Plate 5.1. A).

2. Tissue (4 mm³) was placed in aluminium foil, and immersed in cooled isopentane /liquid nitrogen bath for 2 min, then stored at -70°C until sectioned. When the tissue block was sectioned on a crystat at -20°C, tissue was soft because of isopentane in the tissue which became liquid since the freezing point of isopentane is -150°C. Therefore, it was impossible to cut the sections without destroying the tissue morphology.

3. Tissue (4 mm³) was placed in a small plastic container, filled with Tissue-Tek O.C.T. mounting medium (Miles, Diagnostic Division, Elkhart, IN 46515, USA), and quickly frozen in a precooled isopentane/liquid nitrogen bath. The frozen tissue mounted in the medium was stored in -70°C freezer until processing for immunocytochemistry. When the tissue was frozen by this method, the immunostaining results were greatly improved (Plate 5.1. B). Therefore, this method of freezing tissue, was adopted for the steroid receptor study described in this chapter.

5.2.2. Oestrogen receptor immunostaining

Oestrogen receptor immunostaining was performed according to the method described by manufacturer (ER immunostaining kit from Abbott Laboratories; Diagnostics Division, North Chicago, IL 60064, USA) using the peroxidase anti-peroxidase (PAP) technique and described as follows. After each treatment, the slides were rinsed twice in PBS for 10 min at room temperature expect for step 3.

1. Frozen tissue was sectioned at 5 µm on a cryostat at -20°C, and sections were lifted onto gelatin coated slides and then fixed immediately in 3.7% formaldehyde in PBS for 10 min at room temperature;
2. Fixed sections were then transferred to cold methanol at -20°C for 4 min, followed by cold acetone at -20°C for 2 min;
3. Normal goat serum included in the kit was added as blocking reagent to each specimen and incubated for 30 min at RT;
4. After removing the excess blocking reagent, the specimens from either early or term pregnancy were treated with the primary rat monoclonal oestrogen receptor antibody (0.1 µg/ml) for 16 h at 4°C;
5. The sections were then incubated with goat anti-rat immunoglobulin (1 µg/ml) for 30 min;
6. Finally, the sections were treated with a rat anti-goat gammaglobulin peroxidase-anti-peroxidase complex (0.1 µg/ml) for 30 min at RT.

5.2.3. Progesterone receptor immunostaining

Indirect and PAP immunostaining techniques for progesterone receptor were compared. As the indirect immunostaining method gave much less background, the subsequent experiment was undertaken using this method.

5.2.3.1. Preparation of PAF fixative (pH 7.3)

Based on the method recommended by Stefanini and Perrot-Applanat (Stefanini et al., 1967; Perrot-Applanat et al., 1985), the buffered picric acid-formaldehyde (PAF) fixative was used to fix the tissue for progesterone receptor immunostaining. To prepare PAF fixative, 20g of paraformaldehyde was added to 150 ml of a double-filtered, saturated aqueous solution of picric acid. The solution was heated at 60°C and made alkaline with drops of a 2.52 % (w/v) sodium hydroxide in water to dissociate the paraformaldehyde into formaldehyde. Thus a clear solution was obtained, which was filtered in a cylinder, allowed to cool, and then made up to 1,000 ml with a 0.15 M phosphate buffer.

5.2.3.2. Progesterone receptor immunostaining procedure

1. Frozen sections (5µm) were lifted onto gelatin coated slides, and fixed in PAF for 15 min at -20°C;
2. Fixed sections were then transferred to PBS at 4°C for 40 min before proceeding;
3. Pretreated with 0.5% (v/v) H₂O₂ in PBS for 15 min, followed by rinsing in PBS twice for 10 min at RT;
4. Incubated with 20% (v/v) normal rabbit serum and 5% (w/v) BSA in PBS with 0.15 NaCl for 30 min at RT;
5. After blotting off the excess normal rabbit serum, the sections were then incubated with 5 µg/ml mouse anti-progesterone receptor monoclonal antibody (Transbio; 6 Rue Thiers, 75116 Paris, France) for 30 min for staining progesterone receptor in early pregnancy tissue, and 10 µg/ml for 24 h at 4°C for staining progesterone receptor in term decidua;
6. Rinsed in PBS twice for 10 min;

7. Blocked by normal rabbit serum again for 10 min;
8. Treated with rabbit peroxidase conjugated anti-mouse IgG (1:20 diluted in 20% (v/v) normal rabbit serum) for 30 min at RT.

The immunoprecipitate for the specific antigens (i.e. ER and PR) was visualized by incubation with DAB, which formed a brown reaction product. Sections were lightly counterstained with hematoxylin to facilitate the identification of cellular elements and mounted.

Negative control serial sections, involving substitution of rat or mouse antibody, were performed for each tissue sample.

5.2.4. PRL and progesterone double immunostaining

PRL and progesterone receptor double immunostaining was performed on frozen sections fixed with PAF for 15 min at -20°C . The immunocytochemical localization of progesterone receptor in early decidua parietalis was accomplished by the method described above. The first antigen (progesterone receptor) was visualized by using DAB as the reaction product (brown colour). The sections were then immersed in 1 N HCl for 1 h to elute tissue-bound antibodies. The sections were then blocked by normal swine serum (1:4 diluted in PBS with 5% (w/v) BSA before being exposed to the rabbit anti human PRL; (1:500 diluted in 20% (v/v) normal swine serum with 5% (w/v) BSA) for 30 min at RT. After rinsing in PBS twice for 10 min, biotinylated swine immunoglobulin raised against rabbit immunoglobulin and AB complex were sequentially applied to the sections as described for single antigen localization, followed by rinsing in PBS twice for 10 min after each treatment. The peroxidatic activity of the immunolocally bound AB complex was revealed by incubation in the medium containing 0.1% (v/v) H_2O_2 and 0.3% (w/v) 4-Cl-1-naphthol (Sigma) in 0.05 M Tris/HCl buffer, pH 7.6 (blue colour). The sections stained immunocytochemically in this manner for two antigens were examined without any counterstaining.

The specificity control consisted of substituting normal mouse serum and normal rabbit serum for the specific antisera.

5.2.5. Tissue culture and radioimmunoassay

Organ culture was performed as described in Chapter 4. The culture medium was changed daily for 4-5 days with the spent medium from each day being frozen and stored at -20°C before assaying for PRL using a two-site immunoradiometric assay (IRMA) as described in Chapter 3, and assaying for progesterone using non-extraction radioimmunoassay described in detail as follows:

A specific non-extraction radioimmunoassay was used for measuring progesterone in culture medium according to the method described previously (McNeilly and Fraser, 1987). Briefly, duplicate 50 μl of samples/standards, diluted as required, were added to 100 μl assay buffer (0.1% (w/v) gelatin in phosphate citrate buffer, pH 6.0 with 0.1% (w/v) thiomersal). All tubes apart from NSB then received 100 μl of sheep anti-progesterone (S 361), with subsequent addition of 100 μl [^{125}I]-labelled progesterone (12,000-15,000 c.p.m./100 μl) to all tubes. After incubation at room temperature for 3 h, 100 μl normal sheep serum (1:3200) and 100 μl Donkey anti-sheep serum (1:64) were added to all tubes except for total counts and the tubes were then incubated at 4°C overnight. With addition of 1 ml of 0.9% saline including 4% PEG/0.2% (v/v) Triton-X, tubes were then centrifuged for 30 min at 2,500 rpm at 4°C . The supernatant was discarded and the tubes were then counted in Multigrammer counter.

The reference standard ranging between 7.8-1000 pg/0.1 ml was Preg-4-ene 3, 20-dione (Catalogue No. P0130, Sigma Chemical Co. Ltd). The antibody S361 was used at an initial concentration of 1:10,000. The detection limit was 7.8 pg/tube. The intra- and interassay coefficients of variation were 5.0% and 18%, respectively.

5.2.6. Analysis of data

The changes in PRL and progesterone during organ culture were analysed using paired Student's t test in same group on different days. The changes of different groups on the same day were analysed using unpaired Student's t-test.

5.3. Results

5.3.1. Oestrogen receptor immunostaining

In early decidua parietalis, the nuclei of all of the glandular cells were positively stained for oestrogen receptor, while only a few weakly stained nuclei of decidual cells could be identified (Plate 5.1. C). In decidua capsularis, most of decidualized cells contained oestrogen receptor, and the intensity of staining was much stronger in decidua capsularis than that in decidua parietalis (Plate 5.1. E). The specific staining was always localized in cell nuclei in early decidua, whereas no specific staining in either cytoplasm or nuclei could be identified in term decidua. No nuclear staining occurred in the control preparation, i.e. when a normal non-immunized rat serum was used in place of the anti oestrogen receptor antibody. Term decidual cells did not show any detectable staining of oestrogen receptor in this immunostaining system.

5.3.2. Progesterone receptor

Both decidualized cells in decidual capsularis and parietalis of early pregnancy were stained for progesterone receptor and the staining intensity in decidua capsularis was slightly stronger than that in decidua parietalis (Plate 5.1. F and B). However, no specific progesterone receptor immunostaining was identified in glandular cell of decidual parietalis (Plate 5.1. D). All specific immunostaining was localized in nuclei in early pregnancy decidua. In contrast, the specific progesterone receptor staining was only localized in the cell cytoplasm and not in the nucleus in term pregnancy decidua on either frozen sections (Plate 5.1. I) or dispersed term decidual cells. Indeed, this immunostaining was very faint even using a longer incubation time and a higher concentration of primary antibody. The preparation of term dispersed decidual cells for immunocytochemistry was exactly same as described in Chapter 4. None of the control sections produced any specific staining from either early or term pregnancy decidua.

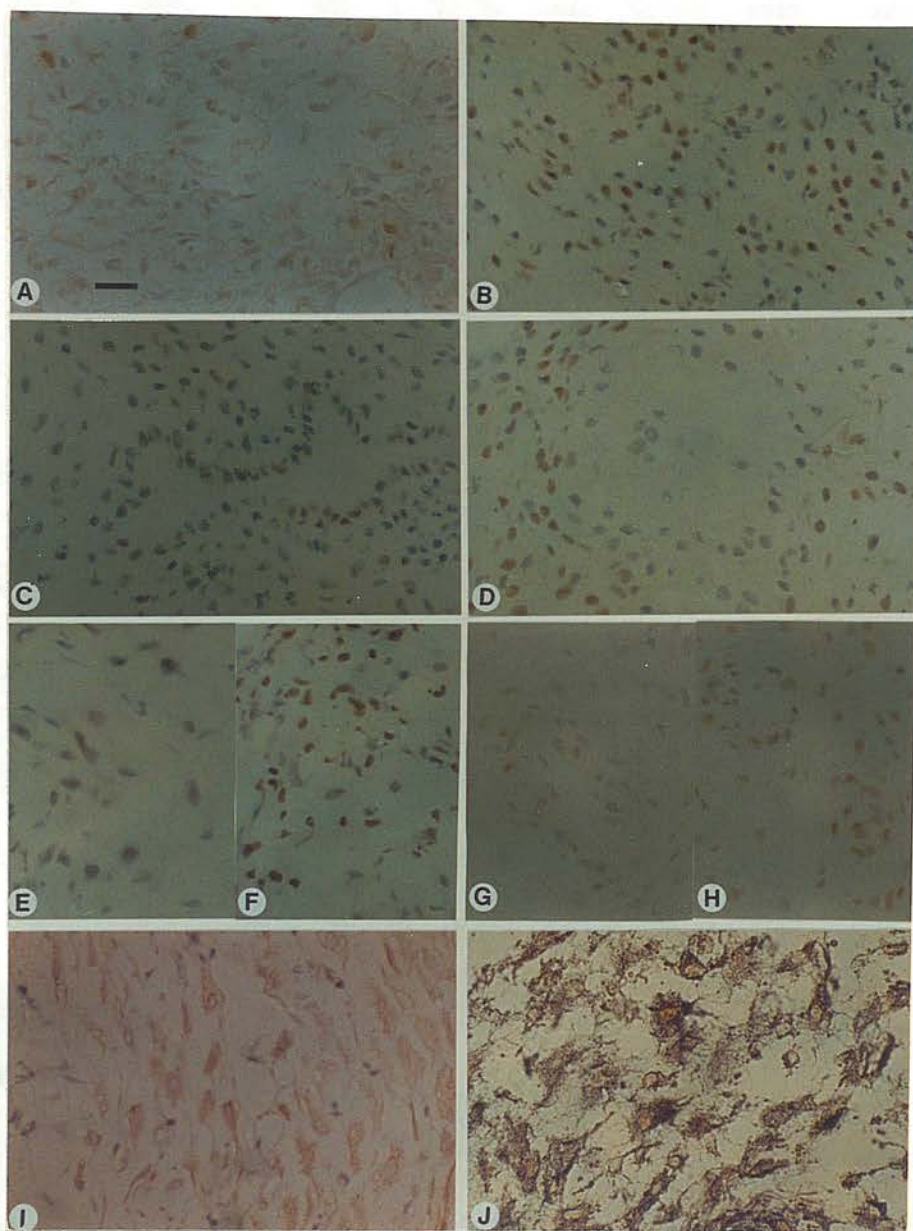


Plate 5.1.

Progesterone and oestrogen receptor distribution in human early and term pregnancy decidua analysed by immunocytochemical method using monoclonal antibodies to human progesterone and oestrogen receptors.

- (5.1.A) Immunolocalization of progesterone receptor in decidua parietalis of early pregnancy. The decidua was frozen in the liquid nitrogen immediately after collection. Note the destroyed integrity of the tissue and very heavy background staining.
- (5.1.B) Immunolocalization of progesterone receptor in decidua parietalis of early pregnancy. The decidua was frozen according to Method 3 of freezing tissue described in the Materials and Methods. Note the morphology of the decidua was much more improved. Progesterone receptor was only localized in the nuclei of the decidual cells without any background staining.
- (5.1.C) Immunolocalization of oestrogen receptor in decidua parietalis of early pregnancy. Immunoreactive oestrogen receptor is only present in the nuclei of epithelial and decidual cells. Much more intense staining is noted in the glandular epithelia than that of decidual cells.
- (5.1.D) Immunolocalization of progesterone receptor in decidua parietalis of early pregnancy. Note the progesterone receptor staining is absent in the nuclei of glandular epithelia in contrast to the strong staining localized in the decidual cells.
- (5.1.E) Immunolocalization of oestrogen receptor in decidua capsularis of early pregnancy. Note decidual cells in decidua capsularis contain higher level of oestrogen receptor than that in decidua parietalis shown in 5.1.C.
- (5.1.F) Immunolocalization of progesterone receptor in decidua capsularis of early pregnancy. Note the nuclear staining in the

Continued....

decidual cells in decidua capsularis is slightly stronger than that in decidua parietalis as shown in 5.1. B and D.

- (5.1.G) Immunolocalization of oestrogen receptor in decidua parietalis of early pregnancy. Note the nuclei of smooth muscle of blood vessels contained oestrogen receptor.
- (5.1.H) Immunolocalization of progesterone receptor in decidua parietalis of early pregnancy. Note the nuclei of smooth muscle of blood vessels contained progesterone receptor.
- (5.1.I) Immunolocalization of progesterone receptor in decidua of term pregnancy. Note progesterone receptor is specifically localized in the cytoplasm in contrast to the absence of the progesterone receptor staining in the nuclei of term decidual cells, suggesting that the nuclei of term decidual cells do not contain progesterone receptor.
- (5.1.J) Double immunolocalization of progesterone receptor and PRL on the same section of decidua parietalis from early pregnancy. Note progesterone receptor is only identified in the nuclei, while PRL is present in the cytoplasm of decidual cells. The positive PRL staining is predominantly associated with these cells containing progesterone receptor in the nuclei.

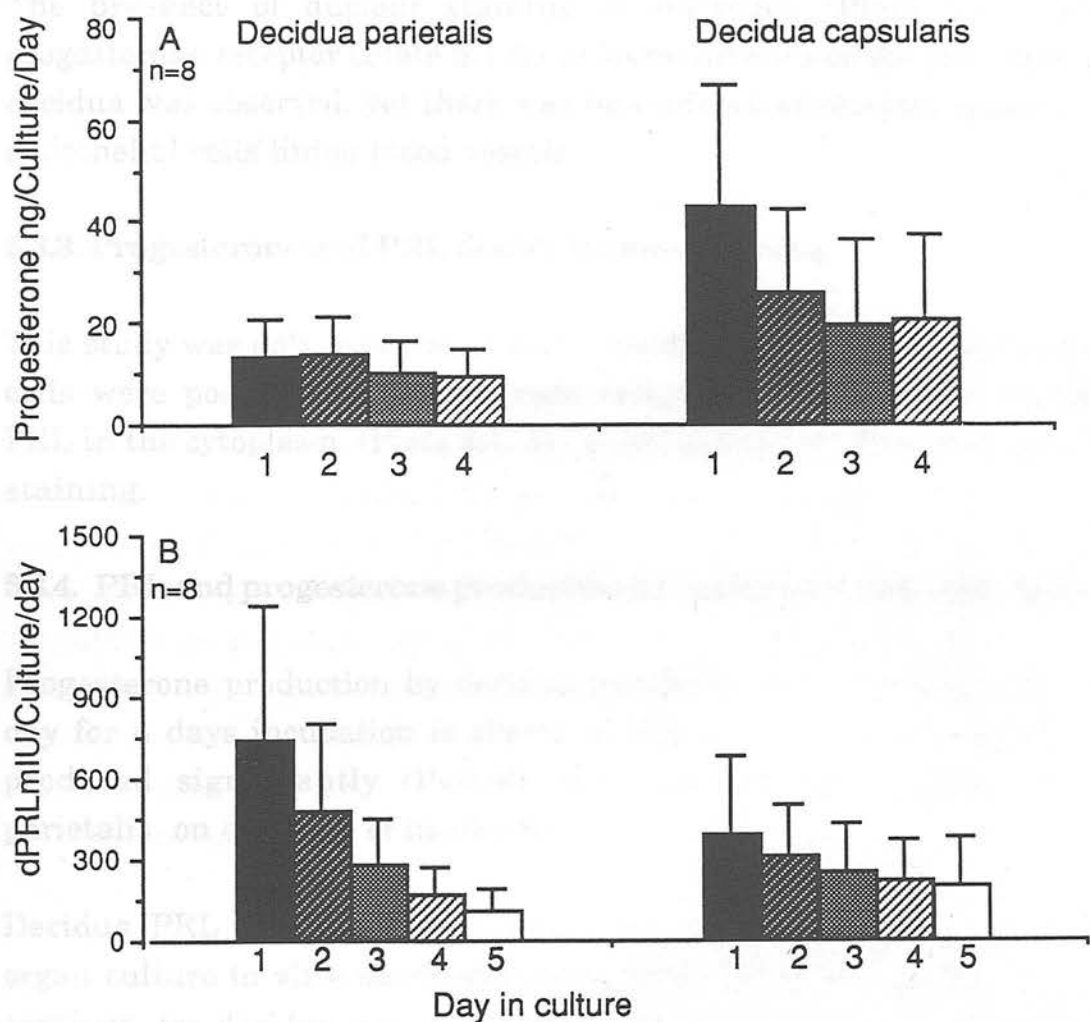


Fig. 5.1. Comparison of progesterone and PRL production in decidua parietalis and capsularis. Tissue was collected from women between days 42 and 56 of pregnancy after therapeutic abortion and cultured in vitro for 4 to 5 days. The amount of progesterone and PRL accumulated in the cultured medium removed from cultures that were prepared from the same sample of decidua ($n=4$ replicates per treatment) were averaged to provide a single representative value for an individual patient ($n=1$). These means were then combined to provide an overall mean \pm s.e.m. for each day of culture in decidua parietalis and capsularis. Note the decidua capsularis produced significantly more progesterone ($P<0.05$) than that in decidua parietalis over each day in vitro culture (A). PRL production in vitro in decidua parietalis declines dramatically ($P<0.05$) in contrast to the maintenance of the PRL level in decidua capsularis upon in vitro culture (B).

The presence of nuclear staining of oestrogen (Plate 5.1.G) and progesterone receptor (Plate 5.1.H) in spiral arteries of the early human decidua was observed, yet there was no evidence of receptor staining in endothelial cells lining blood vessels.

5.3.3. Progesterone and PRL double immunostaining

This study was only executed in early decidua parietalis. Most decidual cells were positive for progesterone receptor in the nucleus, and for PRL in the cytoplasm (Plate 5.1. J). Control sections showed no specific staining.

5.3.4. PRL and progesterone production by decidua in early pregnancy

Progesterone production by decidua parietalis and capsularis on each day for 4 days incubation is shown in Fig. 5.1.A. Decidua capsularis produced significantly ($P<0.05$) more progesterone than decidua parietalis on each day of incubation.

Decidua PRL production by decidua parietalis during the 5 days of organ culture in vitro decreased significantly ($P<0.05$) on each day. In contrast, for decidua capsularis there was a maintenance in decidual PRL production during the 5 day organ culture (Fig.5.1.B).

5.4. Discussion

This is the first study carried out examining steroid receptor distribution in the different regions of early pregnancy decidua. It clearly shows that most decidual cells in either decidua parietalis or capsularis contain progesterone receptors. This provides direct evidence that both decidua capsularis and parietalis are under progesterone control. This suggests that the failure of mifepristone in vivo to cause de-decidualization and subsequent inhibition of PRL production in decidua capsularis was not related to the absence of progesterone receptors. Other factors from trophoblast attached to decidua capsularis play an important role in maintaining decidualization.

Progesterone receptor shifted from epithelial cells to stromal cells, caused either by accumulation of oestrone and oestradiol in the stroma cells (Press et al., 1988) or cumulative activity of progesterone (Garcia et al., 1988). This occurred in the late luteal phase (Press et al., 1988; Garcia et al., 1988), but continued in early pregnancy as demonstrated in the present study, in which no specific progesterone receptor staining was localized in glandular cells. This shift of progesterone receptor to stromal cells coincided with the start of decidualization of stromal cells and PRL production in the late luteal phase. In addition, during pregnancy progesterone mainly acted on stromal cells to maintain decidualization in decidua with increased PRL gene expression (Chapter 4). The results from the present study show for the first time that decidual cells staining positively for PRL always contained progesterone receptor (Plate 5.1. J). These results further indicated that progesterone was a fundamental factor involved in stromal cell decidualization through which expression of PRL gene was promoted. The depletion of progesterone receptor in the glandular epithelium may parallel its declining functional importance in this cell populations during early pregnancy. Stroma, which becomes decidualized, would logically require receptors to support its further growth and differentiation.

Decidual cells are a main target for progesterone during pregnancy and progesterone has been shown to down-regulate the oestrogen receptor (Tseng and Gurpide, 1975; West et al., 1987; Brenner et al., 1990). In addition, treatment with mifepristone in vivo resulted in a large increase in the number of oestrogen receptors in decidua of rhesus macaques during pregnancy (Haluska et al., 1990). Thus the low level of oestrogen receptor in the nuclei of decidual cells in decidua parietalis may be due to an inhibitory action of progesterone acting through the nuclear receptors demonstrated in these cells. The much stronger oestrogen receptor staining in the nuclei of glandular cells may be maintained because there is low or no progesterone receptor present in these cells and thus progesterone would not affect the expression of oestrogen receptor in these cells. However, it remains to be shown whether the high level of oestrogen receptor in glandular cells contributes to a biological effect. In contrast, more intense

staining of oestrogen and progesterone receptor could be identified in decidual cells of decidua capsularis. This may be correlated with the oestrogen produced by trophoblast in decidua capsularis, since it was well established that syncytio-trophoblast produces not only progesterone but also oestrogen. The high local concentration of oestrogen in decidua capsularis could up-regulate its own receptor and the progesterone receptor (Kreitmann-Gimbal et al., 1979; Kreitmann-Gimbal et al., 1980; West et al., 1987), which would, therefore, enhance progesterone action in decidua capsularis to maintain PRL production.

In the present study, decidua capsularis released significantly more progesterone than decidua parietalis which presumably related to the presence of trophoblast cells in capsularis. However, it is unlikely that this endogenous production of progesterone alone can explain the difference in the release of PRL between decidua capsularis and parietalis, as the addition of progesterone alone in vitro could not stimulate PRL secretion in either early or term decidua (Markoff et al., 1983a; Ren and Braunstein, 1990; Chapter 4). It is possible that oestrogen or other factors from trophoblast, together with progesterone participated in modulation of PRL production. This view was further confirmed in the study of term decidual cells in culture in which oestrogen in combination with progesterone modulated PRL production (Chapter 4).

The existence of oestrogen and progesterone receptors in the nuclei of smooth muscles of blood vessels in human decidua, in agreement with previous study (Perrot-Appianat et al., 1988), may relate to a steroid-mediated regulation of uterine blood flow during pregnancy. During pregnancy, blood flow undergoes dramatic increases in order to support fetal growth and homoeostasis (Nuwayhid, 1979; Resnik, 1986). The present immunocytochemistry results imply that sex steroids modulate uterine blood flow through a direct effect on uterine arterial walls.

A striking observation made in the present study was that in term decidual cells progesterone receptor was localized in the cell cytoplasm and not in the nucleus. This is the first report that by using

immunocytochemical technique progesterone receptor was localised in the cytoplasm. Immunocytochemistry of frozen sections most likely revealed antigen in their native localizations, because tissue was processed under low temperature and there was no dehydration through alcohol and xylene which could potentially damage the receptor antigens involved before fixation. It is, therefore, unlikely that nuclear progesterone receptor was translocated into cytoplasm during the preparation of the tissue. Furthermore, progesterone receptors were also present in cytoplasm of the dispersed term decidual cells and the staining for term decidua was performed under same condition as early decidua. Many studies have proved that both unoccupied and steroid-occupied forms of oestrogen and progesterone receptors reside in the nucleus even under conditions of low circulating steroids (Press and Greene, 1984; King and Greene, 1984; Press and Greene, 1988a). Thus, whether this discrepancy of intracellular localization of progesterone receptor in decidual cells between early and term pregnancy resulted from localization of dissimilar forms of steroid receptors remains to be identified. Nevertheless, this cytoplasmic form of progesterone receptor appeared to be functional since progesterone combined with oestrogen could stimulate and maintain PRL production in cultured term decidual cells (Chapter 4). This occurred in spite of the present results in which the oestrogen receptor could not be localized in term decidua. This might relate to the lower concentration of anti oestrogen receptor antibody (0.1 µg/ml) used in this study being insufficient to detect a very low level of oestrogen receptor.

It was suggested by biochemical assay (Padayachi et al., 1987) and confirmed in this study that with advancing of gestation, decreases in oestrogen and progesterone receptors became more marked. This was presumed to be due to the down-regulating effect of increased progesterone throughout pregnancy on both steroid receptors as seen in the luteal phase of menstrual cycle and in patients treated with the combined oral contraceptive pill (Illingworth et al., 1971).

Combining the previous studies from other groups and the serial investigations of steroids and their receptors in this thesis, several assumptions can be drawn: progesterone was required for induction

and maintenance of decidualization in the human endometrium, thus switching on PRL production (Daly et al., 1983a; Maslar and Ansbacher, 1986) in the normal menstrual cycle. This extended throughout pregnancy, since there was increased expression of PRL mRNA within cells as decidualization progressed in pregnancy (Chapter 4 and 5). Moreover, the double progesterone and PRL immunostaining results further support the concept discussed above, that specific PRL staining was only associated with the cells which contained progesterone receptors. Furthermore, progesterone was only capable of inducing and maintaining decidualization in oestrogen-primed tissue since in the present study progesterone could modulate PRL production only when used together with oestrogen in vitro. As progesterone receptors are oestrogen-dependent, there may be an indirect association between oestrogen and progesterone receptors at receptor level. This mechanism may be involved in controlling PRL production between decidua parietalis and capsularis and cultured term decidual cells treated by steroids, with the combination of oestrogen and progesterone produced by trophoblast in decidua capsularis or added in culture medium, maintaining progesterone receptors and modulating PRL production.

Apart from steroids, other potential factors from trophoblast, e.g. placenta derived releasing factor (Handwerger et al., 1987a), IGF-1 (Thraifill et al., 1988) serving as mediators of the effects of sex steroids (Brenner et al., 1990) or as direct regulators, may also play a role in the differentiation of decidual cells and maintenance of PRL production.

In summary, these data suggest that while progesterone induced and maintained PRL production via its receptor mechanism as result of decidualization, oestrogen may modulate progesterone function at receptor level, thus being indirectly involved in modulating PRL production. This interplay between progesterone and oestrogen modulated PRL production in vivo throughout pregnancy. Whether or not other releasing factors from trophoblast are involved in this process is open to further study.

Local Control of Decidual Prolactin Production by Non-steroid Factors

6.1. Introduction

Earlier observations in Chapter 4 have shown that sex steroids may be the potential modulators for decidual PRL production. However, the maintenance of the decidualization during pregnancy is poorly understood, and it has been suggested that in normal pregnancy blastocyst-induced decidualization probably augments that initiated by sex steroids of the cycle (Finn, 1977). The next step in the present study was to investigate whether factors from feto-placental unit were directly involved in decidualization and PRL production during human pregnancy. In addition, little was known about the intracellular mechanisms involved in the control of release of PRL from human decidua tissue. The aims of the following studies were to investigate, firstly, the potential PRL releasing activity associated with the placenta and secondly, the intracellular signal transduction factors affecting decidual PRL production. Thus the effects on decidual PRL production of cAMP and TPA were assessed to determine the potential input of the adenylate cyclase and phosphoinositol second messenger pathways in the control of PRL synthesis and release.

6.2. Materials and Methods.

Early pregnancy tissues between 7-11 weeks of gestation from therapeutic abortions were received fresh in the theatre where they were isolated into decidua and placenta and put into a sterile bottle containing RPMI-1640 medium supplemented with 5 $\mu\text{g}/\text{ml}$ gentamycin and 2 $\mu\text{g}/\text{ml}$ amphotericin B for transporting to the laboratory. The term decidua and placenta from uncomplicated pregnancies between 37-40 weeks were obtained in the labour ward and then transferred to the laboratory in the same way. The organ culture and cell culture were performed exactly as described in Chapter 4.

6.2.1. Experimental design

In all these studies the putative releasing or inhibiting substances were tested for their ability to alter the production of PRL from both early and term decidua maintained in organ cultures as described in Chapter 4.

6.2.1.1. Early or term placenta conditioned medium (PCM)

To prepare placental conditioned medium, human early or term placenta cut into 5 mm³ was incubated overnight in RPMI-1640 medium (1g placenta/ 15 ml medium). The medium defined as PCM was then collected by discarding the placenta tissue and stored at -20°C until used in the culture system.

Both early and term pregnancy decidua were included in the experiments using organ culture and the acute response (1 h) and chronic response (24 h) of decidual PRL secretion to 10%, 30%, 50% PCM respectively were then tested.

To test the acute response, after preincubation of decidua tissue in control medium (RPMI-1640 medium supplemented with 5% charcoal stripped calf serum, 50 µg/ml gentamycin and 2 µg/ml amphotericin B) for 1 h, the medium was changed to control medium or medium containing 10%, 30%, or 50% PCM prepared from early or term placenta for an additional 1 h. The acute effect of PCM was tested on different decidua from early (n=4) and term (n=7) pregnancies. The spent medium from both the preincubation and 1 h incubation was collected and stored at -20°C until assayed for PRL as described in Chapter 4.

To test the chronic response of decidua to PCM, decidua from either early (n=5) or term (n=3) pregnancies was treated with variable concentrations of PCM. After 24 h incubation, the experiment was terminated by collecting medium and the spent medium was stored at -20°C for measurement of PRL. All experiments were carried out with 4 replicates per treatment.

6.2.1.2. Human chorionic gonadotropin (hCG)

Early or term decidua explants in in vitro culture were exposed to hCG (Intervet UK Ltd., Science Park, Milton Road, Cambridge CB4 4FP, UK) at the concentrations of 0.1, 1 or 10 U/ml medium for 3 days with the medium changed daily. Decidua from 6 early and 9 term pregnancies was included in this study with 4 replicates for each treatment from each tissue. The acute response (1 h) of decidual PRL secretion to hCG was only tested in term decidua from 4 patients.

In addition, term dispersed decidual cells from 3 different tissues were examined for the chronic response of decidual PRL secretion to hCG at the same concentrations as above for 3 days. Both organ and cell culture were performed as described in chapter 4. The cells were plated at the density of $0.5-1 \times 10^6$ /ml with the medium renewed daily.

6.2.1.3. cAMP analysis

Term decidual cells from 5 different tissues were prepared as described in Chapter 4 and plated at a density of 2×10^6 /ml. After 48 h incubation, the medium was removed and the cells washed in serum-free RPMI-1640 medium twice and incubated for additional 2 h in serum-free medium. At the end of 2 h incubation, 0.2 ml of medium was aspirated from each well, replaced with control medium (serum-free) alone or medium containing dibutyryl cAMP (Sigma, Lot No 89F-7180), which was dissolved directly into medium immediately prior to each experiment at the concentrations of 0.1 mM, 1 mM, or 5 mM. Incubation was continued for 0.5 h and 1 h. Each treatment had 4 replicates. Decidua explants exposed for 1 h to the medium containing 10%, 30%, and 50% PCM released 39%, 83%, and 152% more PRL. At the end of each experiment, the medium was removed and to determine PRL cell content the cells were washed twice with PBS, then resuspended in 0.1%(v/v) Triton X-100 (500 μ l per well) for 15 min with or without being freeze-thawed three times. The cell suspensions were then centrifuged for 0.5 h at 2600 x g. The cell suspensions and media were frozen at -20°C until PRL was measured by radioimmunoassay as described in chapter 4.

6.2.1.4. Phorbol ester 12-0-tetradecanoyl phorbol 13-acetate (TPA)

Term decidual cells from 3 different tissues were prepared and plated at the density of $0.5-1 \times 10^6/\text{ml}$, then exposed to TPA (Sigma chemical Co., UK) at 10^{-6} to 10^{-12} M (triplicate per treatment) for 3 days. Medium was changed daily and the media were stored in -20°C for later assay of PRL.

In one set of experiments, the effect of oestrogen and progesterone, alone or together, on the PRL response to TPA was examined. The cells were exposed to sex steroids alone for the first 48 h after cells were plated. On the third day of culture cells were exposed to the sex steroids in the presence or absence of TPA. The cells were then incubated for another 3 days with media contained steroids and TPA replaced daily. Decidual PRL levels were measured in the collected cultured medium.

6.2.2. Statistical analysis

Four replicates of each treatment from one tissue were averaged to one mean ($n=1$) and then an overall mean calculated for each treatment from the different tissue depending on the experiments. Statistical analysis was performed using analysis of variance. Contrasts between different groups were performed using Duncan's multiple range test.

6.3. Results

6.3.1. The effect of PCM

Term decidua explants exposed for 1 h to the medium containing 10%, 30%, and 50% PCM released 39%, 83%, and 152% more PRL, respectively, than explants exposed to control medium alone. (Fig. 6.1. A). This increase was only significant ($p < 0.05$) after the addition of 50% PCM.

In contrast, there was no acute stimulation of PRL release from decidua when the early decidua was incubated with 10%, 30% or 50%

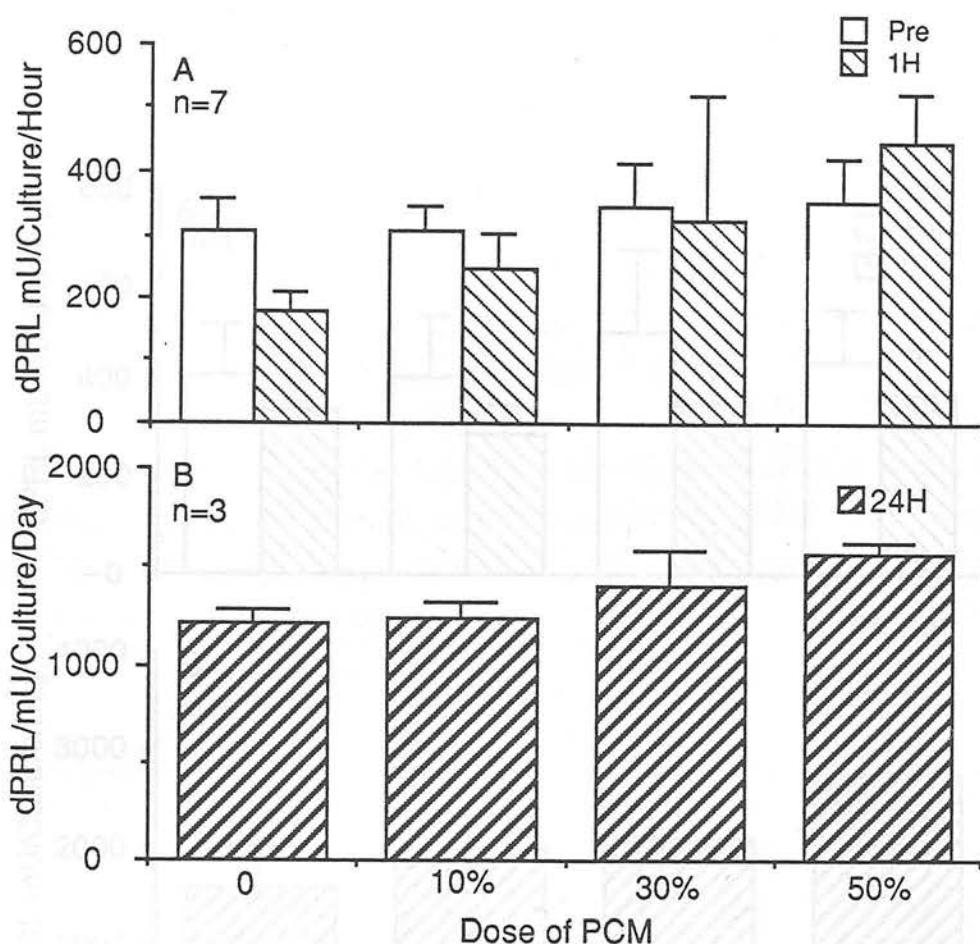


Fig. 6.1. The effect of placenta conditioned medium (PCM) on the release of decidual PRL from term decidua. Four replicate cultures of decidual explants were exposed to the culture medium (control) or medium containing 10%, 30% and 50% PCM. The results are expressed as the mean \pm s.e.m. The top panel (A; $n=7$) shows the acute response of decidual PRL to PCM. After 1 h preincubation in the medium without supplement of PCM, decidual explants were incubated at various doses of PCM for additional 1 h. The decidual explants released 39%, 83% and 152% more PRL in response to 10%, 30% and 50% PCM. This increase was only significant ($P<0.05$) after the addition of 50% PCM. The bottom panel (B; $n=3$) shows the amount of PRL released by term decidua exposed to 10%, 30% and 50% PCM for 24 h. In each instance, there is no significant increase ($P>0.05$) in the amounts of PRL released by PCM treated decidua compared with that received control medium only.

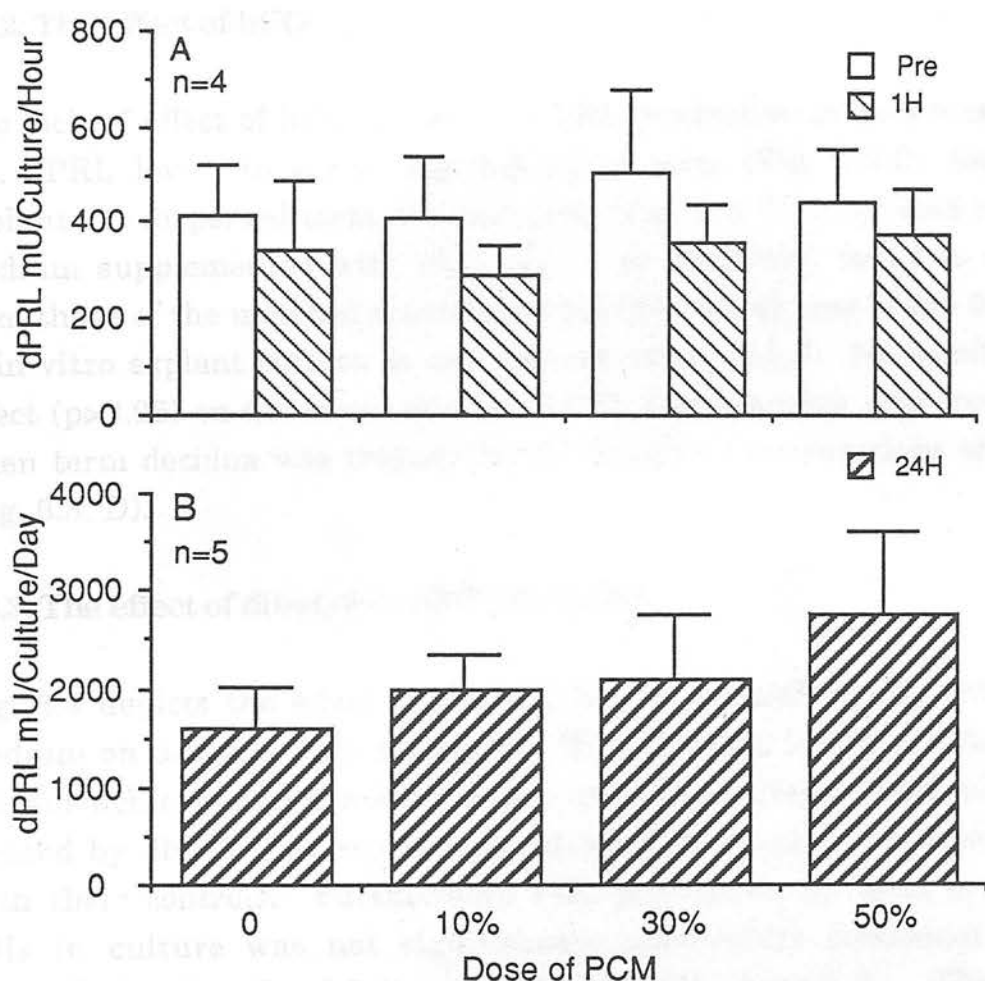


Fig. 6.2. The effect of PCM on the release of PRL from early pregnancy decidua. Four replicate cultures of decidual explants were exposed to either the control medium or medium containing 10%, 30% and 50% PCM. The results are expressed as the mean \pm s.e.m. Exposure of early decidua to PCM either for 1 h (A; n=4) or 24 h (B; n=5), had no significant effect on the amount of PRL released by early decidua.

PCM for 1 h (Fig. 6.2. A). In addition, chronic exposure of either early or term decidua to PCM for 24 h had no significant effect ($p>0.05$ in each instance) on release of PRL, although there was a trend for an increase in PRL release in both experiments (Fig. 6.1.B and Fig. 6.2.B).

6.3.2. The effect of hCG

The lack of effect of hCG on decidual PRL production is shown in Fig. 6.3. PRL levels in early (Fig. 6.3.A), or term (Fig. 6.3.B) decidua explants or dispersed term decidual cells (Fig. 6.3. C) incubated in the medium supplemented with hCG (0.1, 1 or 10 IU/ml) failed to differ from those of the matched control decidua ($p>0.05$) on any of the 3 days of in vitro explant culture at any concentration tested. No significant effect ($p>0.05$) on the acute release of PRL from decidua was observed when term decidua was treated by the variable concentrations of hCG (Fig. 6.3. D).

6.3.3. The effect of dibutyryl cAMP (dbcAMP)

Fig. 6.4 depicts the effect of the addition of dbcAMP to the cultured medium on decidual PRL secretion. The difference in PRL production did not achieve significance ($p>0.05$) on cultured term decidual cells treated by dbcAMP at any concentration of 0.1, 1 or 5 mM compared with their controls. Furthermore PRL production by term decidual cells in culture was not significantly affected by treatment with dbcAMP in vitro for 0.5 h and 1 h (Fig. 6.4. A and B). The PRL concentration in the cell suspensions was undetectable (<50 mU/ 10^6 cells).

6.3.4. The effect of TPA

The effect of TPA on the release of PRL from human term decidual cells in culture is shown in Fig. 6.5. A (Day 1), B (Day 2) and C (Day 3). Triplicate cultures of decidual cells from 3 different tissue were exposed to control medium or medium containing TPA at different concentrations. On each day of the 3 days in in vitro culture, TPA at the concentrations of 10^{-8} to 10^{-5} M inhibited the PRL production

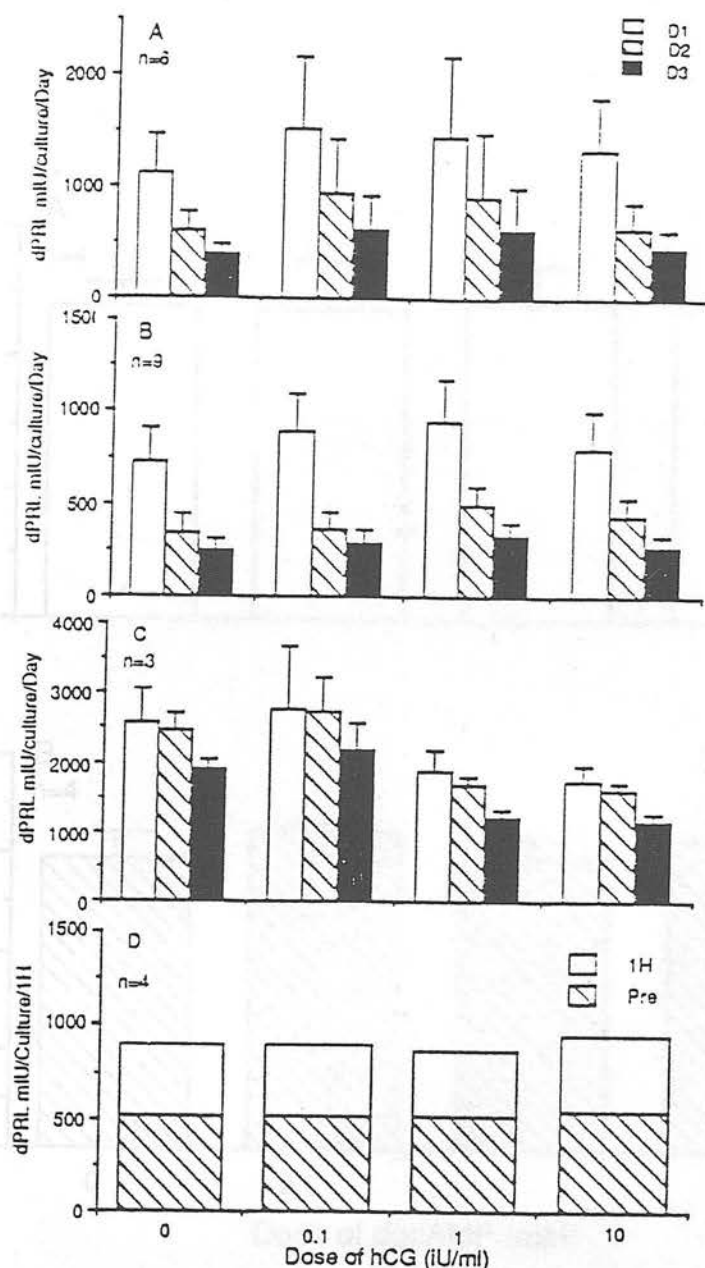


Fig. 6.3. The effect of hCG on the release of PRL from early and term decidua. Four replicate cultures of decidual explants were exposed to either the control medium or medium containing 0.1-10 IU/ml hCG. The results are expressed as the mean \pm s.e.m. hCG had no effect on decidual PRL release in either early pregnancy (A; n=6) or term pregnancy (B; n=8) during a 3 day in vitro organ culture. The same negative effect of hCG on decidual PRL production was observed in the cultured term decidual cells (C; n=3) during a 3 day in vitro organ culture. When term decidua explants were exposed to hCG for 1 h (D; n=4), again, no any change in the amount of decidual PRL measured in the cultured medium was noticed in presence of hCG.

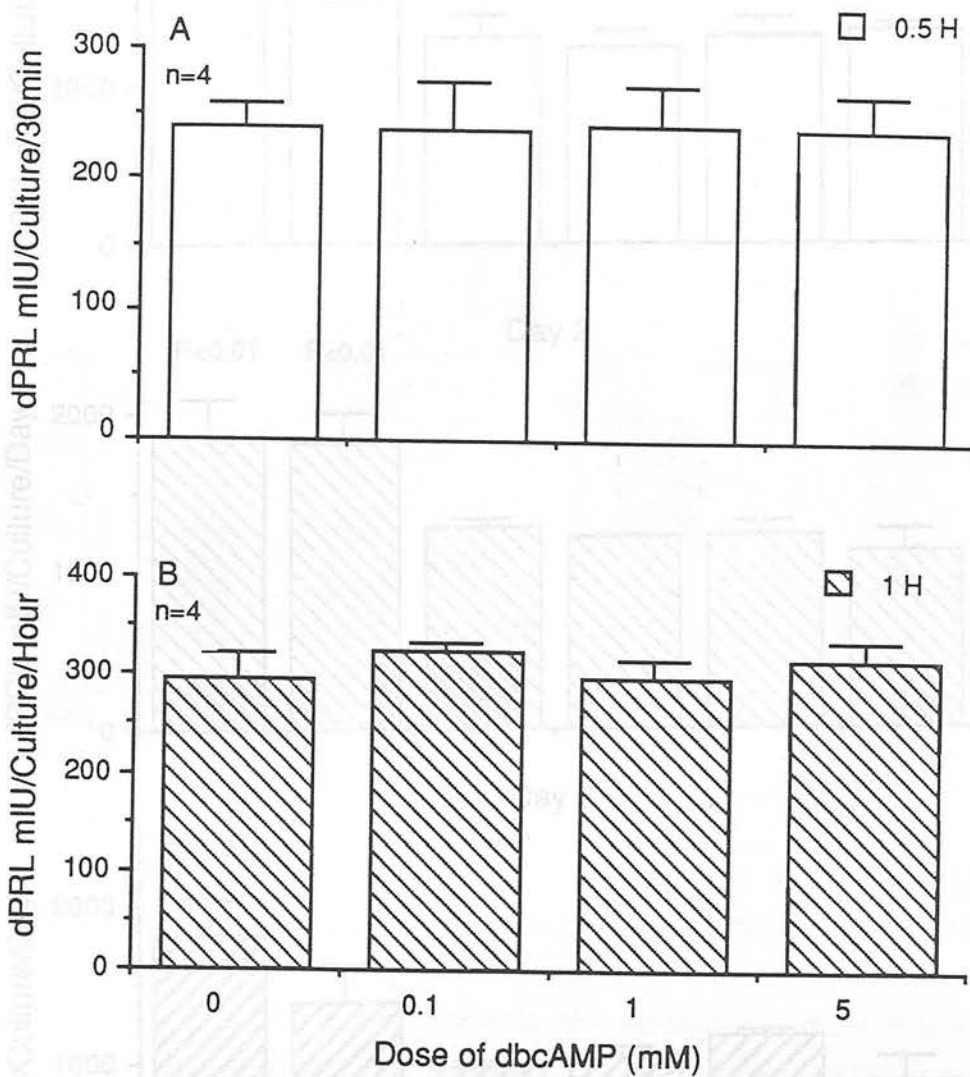


Fig. 6.4. The effect of dbcAMP on the release of PRL from term decidual cells. Four replicate cultures of term decidual cells were exposed to either the control medium or medium containing 0.1-5 mM dbcAMP. The results are expressed as the mean \pm s.e.m. The amount of PRL released by the term decidual cells exposed to dbcAMP for either 0.5 h (A; n=4) or 1 h (B; n=4) was not significantly different from that exposed to the control medium only.

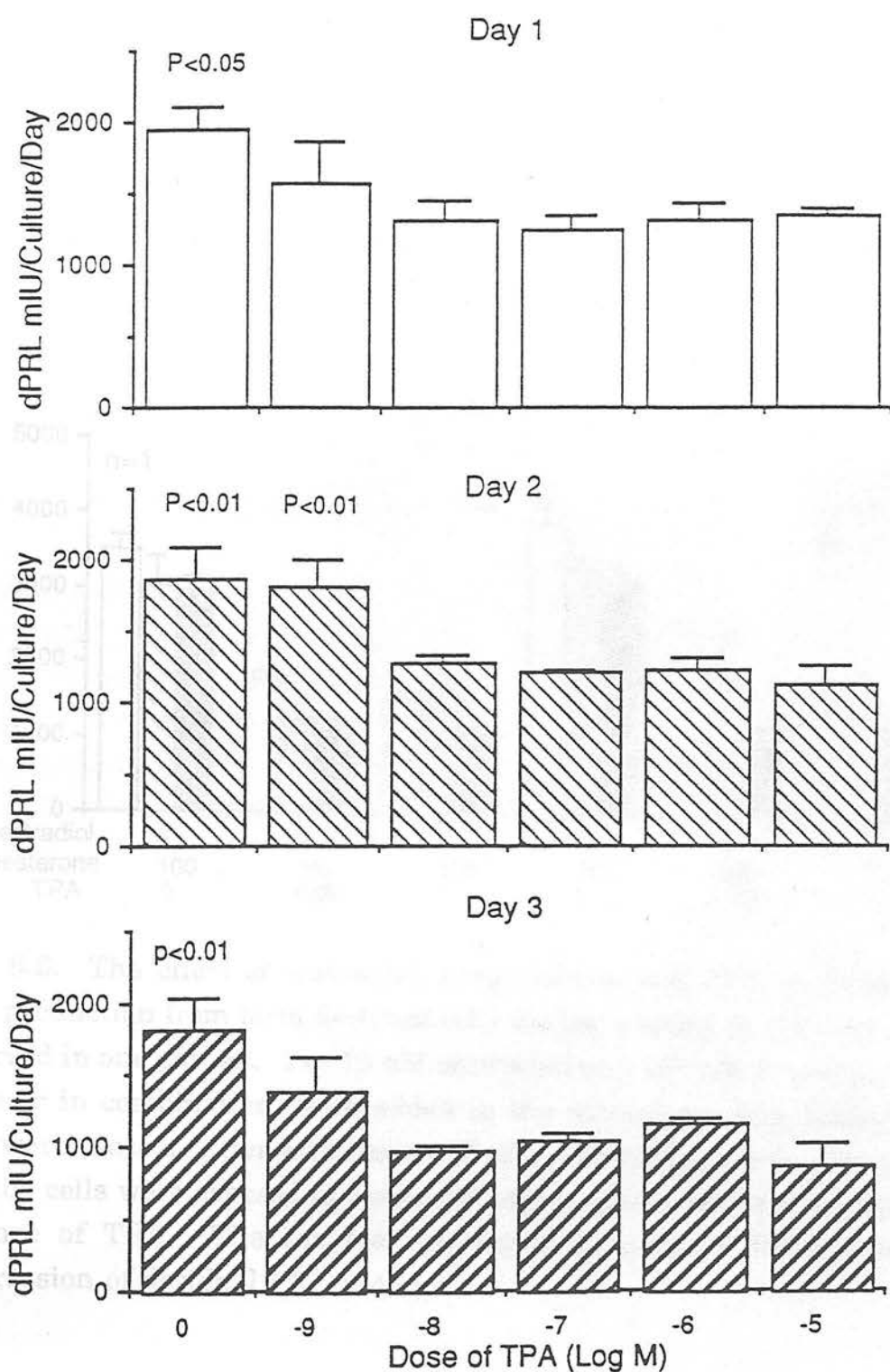


Fig. 6.5. The effect of TPA on the release of PRL from term decidual cells during a 3 day in vitro culture. Triplicate cultures of term decidual cells were exposed to either the control medium or medium containing TPA at the concentrations between 10^{-5} - 10^{-9} M. On each of the three days (A-C; $n=3$) of in vitro culture, TPA at concentrations of 10^{-8} to 10^{-5} M inhibited the PRL production significantly ($P < 0.05$ on day 1, $P < 0.01$ on day 2 and 3).

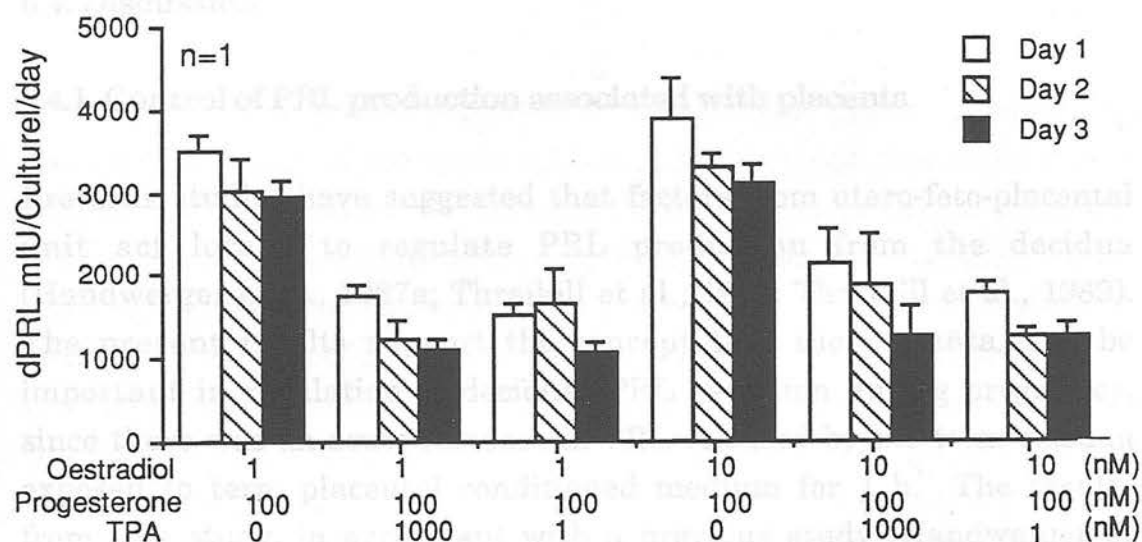


Fig. 6.6. The effect of oestradiol, progesterone and TPA on decidual PRL production from term decidual cells during a 3 day in vitro culture observed in one patient. 1 or 10 nM oestradiol and 100 nM progesterone, alone or in combination, were added to the culture medium when the cells were plated. After attachment of cells, the medium were changed and the cells were exposed to oestradiol and progesterone in presence or absence of TPA. The sex steroids failed to prevent TPA-induced suppression of decidual PRL production.

significantly ($p < 0.05$ on day 1, $p < 0.01$ on day 2 and 3) in a time and dose-dependent manner with maximal inhibition at the concentration of 10^{-5} M on day 3.

The addition of oestradiol at either 1 or 10 nM together with progesterone at 100 nM failed to prevent the TPA-induced reduction in PRL production (Fig. 6.6.).

6.4. Discussion

6.4.1. Control of PRL production associated with placenta

Previous studies have suggested that factors from utero-feto-placental unit act locally to regulate PRL production from the decidua (Handwerger et al., 1987a; Thraikill et al., 1988; Thraikill et al., 1989). The present results support the concept that the placenta may be important in regulation of decidual PRL secretion during pregnancy, since there was an acute increase in PRL released by the term decidua exposed to term placental conditioned medium for 1 h. The results from this study, in agreement with a previous study (Handwerger et al., 1983), confirmed that the placenta released a factor(s) that stimulated the release of PRL. However, this PRL releasing activity associated with the placenta was reduced or absent when the term decidua was exposed to placental conditioned medium for 24 h. This contrasts with previous reports in which a factor derived from placenta induced a biphasic stimulation, i.e. acute and chronic stimulation of PRL secretion and synthesis (Golander et al., 1988).

An earlier purification study suggested that the PRL releasing factor(s) was heat (56°C for 30 min) and pH (3.0-10.0) stable, unaffected by lipid extraction, sensitive to proteolytic digestion and appeared to be a single protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an apparent molecular weight of 23.5 K (Handwerger et al., 1987a). However, the nature of this placentally derived factor has still not been determined.

It was of interest to note that neither acute nor chronic release of PRL

from decidua was affected in the early pregnancy decidua incubated with early placental conditioned medium for either 1 h or 24 h. Because both early and term experiments were performed under exactly the same conditions, the failure of stimulation of PRL release from early pregnancy decidua indicated that this PRL releasing factor(s) probably did not participate in the regulation of PRL production in early pregnancy. The dynamic pattern of the placentally derived releasing factor in modulating PRL secretion throughout human pregnancy still needs to be defined.

Human chorionic gonadotropin (hCG) is the major protein produced by the langhans cells of the cytotrophoblast in the large quantities during pregnancy. It was logical to inquire whether this major placental protein was involved in regulating PRL release and synthesis from decidua. Thus extensive experiments were carried out in this study to examine the effect of hCG on PRL production by decidua. Neither early nor term decidual explants responded to hCG in terms of PRL production, this being further confirmed in term decidual cell culture. A single study from Rosenberg and Bhathagar (1984) demonstrated that HCG at a specific dose (10^4 mIU/ml) stimulated PRL production in in vitro organ culture of term decidua. However, this result was disputed by other reports in which either hCG given repeatedly in anovulatory monkeys (Ying et al., 1988) or added to culture medium in vitro (Handwerger et al., 1983) failed to induce or stimulate PRL production, consistent with the present study. As the hCG levels range between 4,000-11,000 IU per litre in the second and third trimesters of pregnancy, the concentration of hCG tested in the present study covered the physiological ranges in the serum of pregnancy. The present results do not support any role for hCG in regulating PRL production during pregnancy.

6.4.2. Intracellular signalling factors regulating decidual PRL production

Unlike steroid hormones which bind to specific hormone receptors in the cytoplasm or nucleus and then directly activate transcriptional enhancers located in DNA sequences, all water soluble signalling

molecules including protein hormones bind to specific receptor proteins on the surface of the target cells and convert this extracellular event into signals inside cells. Hormone release in most endocrine tissues appears to be triggered by the G-protein linked receptor mechanism, which activate or inactivate adenylate cyclase, thereby altering the intracellular cAMP concentration, or phospholipase-C which generates either inositol triphosphate (Ins P₃) to increase Ca⁺² concentration, or diacylglycerol (DAG) to activate protein kinase C (Alberts et al., 1989).

Extensive studies have shown that the regulation of synthesis and release of decidual PRL differs from pituitary PRL, but failure of a number of hormones to affect PRL secretion from decidual cells could be explained by a lack of the corresponding receptors in the decidual cell. The intracellular messenger system controlling PRL production is not well defined at present. The current finding has demonstrated that dibutyryl cAMP, an analogue of cAMP, in the concentration between 0.1 to 5 mM had no effect on PRL secretion of decidual cells in either 0.5 h or 1 h in vitro exposure to cAMP. These results are in apparent contradiction with previous reports (Handwerger et al., 1987b) in which an increase in intracellular cAMP inhibited the releasable PRL.

Another second messenger system investigated in this study is protein kinase C. The activation of protein kinase C can be achieved by diacylglycerol or phorbol esters (TPA). It was clearly shown in this study that TPA incubated with decidual cells in vitro for 3 days inhibited PRL production significantly in a time and dose dependent manner. The time course of TPA inhibition in decidual PRL is consistent with a role for protein kinase-C in altering PRL synthesis. The inhibitory effect of TPA on decidual PRL existed even in the presence of sex steroids, which indicated that the effect of TPA was to negate the positive effect of oestradiol and progesterone on decidual PRL synthesis. To our knowledge, this represents the first report of a long-term action of TPA on decidual PRL production. A similar inhibitory effect of TPA in a long term action on PRL synthesis has been reported in the human B-lymphoblastoid cell line IM-9-P3 which represents

another ectopic source of human PRL (Gellersen et al., 1989b). This PRL resembles decidual PRL according to its similar molecular size of PRL mRNA (Gellersen et al., 1989a), identical 5' untranslated gene structure (DiMattia et al., 1990), very low intracellular storage and the unresponsiveness to many secretagogues controlling pituitary PRL secretion (Gellersen et al., 1989b).

Although decidual PRL has chemical and biological properties identical to those of pituitary PRL, the regulation of release of PRL from decidua is distinct from that of pituitary PRL, even at the second messenger level. PRL secretion in the pituitary is stimulated by the protein kinase C signal transduction mechanism at the level of a readily releasable intracellular PRL pool as well as at the level of gene transcription and protein synthesis (Oshorne and Tashjian, 1981; Maurer, 1982; Murdoch et al., 1985). However, the opposite, inhibitory effect of activation of protein kinase C signal pathway on decidual PRL production was observed in the present study. In addition, activation of the adenylate cyclase-cAMP system had no effect on decidual PRL secretion, again in contrast to its stimulative effect on the pituitary PRL release (Tam and Dannies, 1981; Maurer, 1982). Marked differences in control of PRL secretion between pituitary and decidua might be associated with the different intracellular storage amount and sites, in that decidual PRL has a very small intracellular pool confirmed in the present study and no particular subcellular storage site (Handwerger et al., 1984), while pituitary PRL is stored in large secretory granules.

Taken together, the present results support the concept that complex regulatory mechanisms for decidual PRL production might exist, operating at the local level associated with placenta, but hCG appeared to be not involved in the control of PRL production by decidua. In terms of the intracellular transduction signal system, unlike the pituitary PRL, an increase of intracellular cAMP had no effect on release of decidual PRL, whereas the activation of protein kinase C by chronic exposure to TPA inhibited the production of PRL.

Effect of Dopamine on Decidual Prolactin Production before and after Transfection of Decidual Cells with Dopamine D2 Receptor

Part I. Localization of Dopamine D2 Receptor Gene Expression in Human Decidua

7.1.1. Introduction

It has been well-established that dopamine is a major physiological inhibitor of PRL secretion that directly acts on the pituitary gland. In addition, dopamine receptors have been localized on the plasma membrane of pituitary cells, and there is a good correlation between the relative binding affinities of dopamine and the inhibition of PRL secretion (Caron et al., 1978). Thus it is likely that dopaminergic inhibitory effect on pituitary PRL is mediated through dopamine receptors. In contrast, dopamine or its agonist, bromocriptine, failed to affect PRL secretion by human decidua (Golander et al., 1979; Ben-Jonathan et al., 1980). It is not clear whether this is due to a failure of dopamine to affect PRL after binding to its receptor, or because no dopamine receptor is present in the decidual cells. In view of the major difference in dopaminergic control of PRL release in decidua compared to the pituitary, the effect of the dopamine agonist, bromocriptine, on PRL production by human term pregnancy decidual cells in culture was reexamined in the presence and absence of oestradiol and progesterone shown previously (Chapter 4) to influence decidual PRL production. The potential presence of dopamine receptors in human decidual cells was then examined by in situ hybridization, Northern blot analysis and polymerase chain reaction (PCR). Finally, decidual cells in culture were transfected with a dopamine D2 receptor construct to increase expression of dopamine receptor and determine whether dopamine could then act to control decidual PRL production.

7.1.2. Materials and methods

7.1.2.1. Collection of human decidua, rat and sheep pituitaries

Human term placentae with membranes between 37-40 weeks of gestation from uncomplicated pregnancies were obtained in the labour ward where decidua was stripped from chorion, rapidly frozen and stored in liquid nitrogen for later extraction of RNA. Dispersed term decidua cells prepared as described in Chapter 4 were used for the in situ hybridization study. After attachment of cells to the slides the cells were washed twice with RPMI-1640 medium and then stored at -70°C .

Both rat and sheep pituitaries known to express dopamine receptor shown by in situ hybridization (Peters et al., 1991) were used as positive controls for in situ hybridization and PCR. Female adult rats were maintained in an individual cage in a room with controlled light (14 h of light, 10 h of darkness) and temperature ($22-24^{\circ}\text{C}$) and fed on Purine rat chow and tap water ad libitum. They were killed by decapitation, the pituitaries removed within 1 min, washed in cold PBS before they were frozen in dry ice and stored in -70°C freezer until use. Sheep pituitaries were removed within 2 min after they were killed with an overdose of sodium pentobarbitone and processed as for the rat pituitaries. Sheep pituitaries were used as a positive control for Northern blot analysis.

7.1.2.2. Treatment of decidual cells with bromocriptine

Decidual cells from 4 term pregnancy tissues were treated with bromocriptine at the doses 1×10^{-6} , 1×10^{-7} , 1×10^{-8} , and 1×10^{-9} M for 3 days during in vitro culture. Each treatment had 4 replicates. At the end of experiment, the cultured medium was collected and stored at -20°C until assayed for PRL.

In a second set of experiments, oestrogen at 1 nM and 10 nM and progesterone at 1 nM and 100 nM were added separately or in combination to the culture medium when cells were plated. After 48 h incubation, the cells were then treated with bromocriptine at 10^{-6} M in

the continued presence of the same concentration of oestradiol and progesterone.

7.1.2.3. In situ hybridization using ribo-probes

A. Preparation of ribo-probes

The plasmid containing rat dopamine D2 receptor cDNA cloned in the E. coRI site of pBluescript was generously provided by Dr. K. Eidne, MRC Reproductive Biology Unit. The clone contained a 1.5 kilobase insert that coded from the third cytoplasmic loop to the 3' poly A tail of dopamine D2 receptor (Fig. 7.1.) and was used for constructing ribo-probes for the in situ hybridization study. The plasmid dopamine D2 receptor cDNA was prepared using a Miniprep kit (Pharmacia LKB, Biotechnology; see **Appendix 9**). After purification the plasmid was linearized by restriction enzymes (Xba 1 and Kpn 1), which cleaved downstream from inserted sequences, with respect to the T7 and the T3 promoters, forming the DNA template (see **Appendix 10** for restriction enzyme procedure). T7 (antisense) or T3 (sense) RNA transcripts were synthesized and labelled with [³⁵S] UTP in vitro using the mCAP transcription kit (see **Appendix 11** for ribo-probe labelling method) to a specific activity 1-2 x 10⁶ c.p.m./μl. The antisense probe hybridized to the cellular mRNA and the sense riboprobe acted as a negative control. The radioactive ribo-probes were then hydrolysed for 70 min at 60°C to produce a shorter probe (100-120 bp) to facilitate penetration of the probe into the tissue.

B. In situ hybridization procedure

Frozen sections of rat pituitary (5 μm) were cut at -20°C on a cryostat, and floated on to the precleaned poly-L-lysine coated slides. The slides with cultured decidual cells were removed from the -70°C freezer and dried quickly, together with frozen sections of rat pituitary, and then

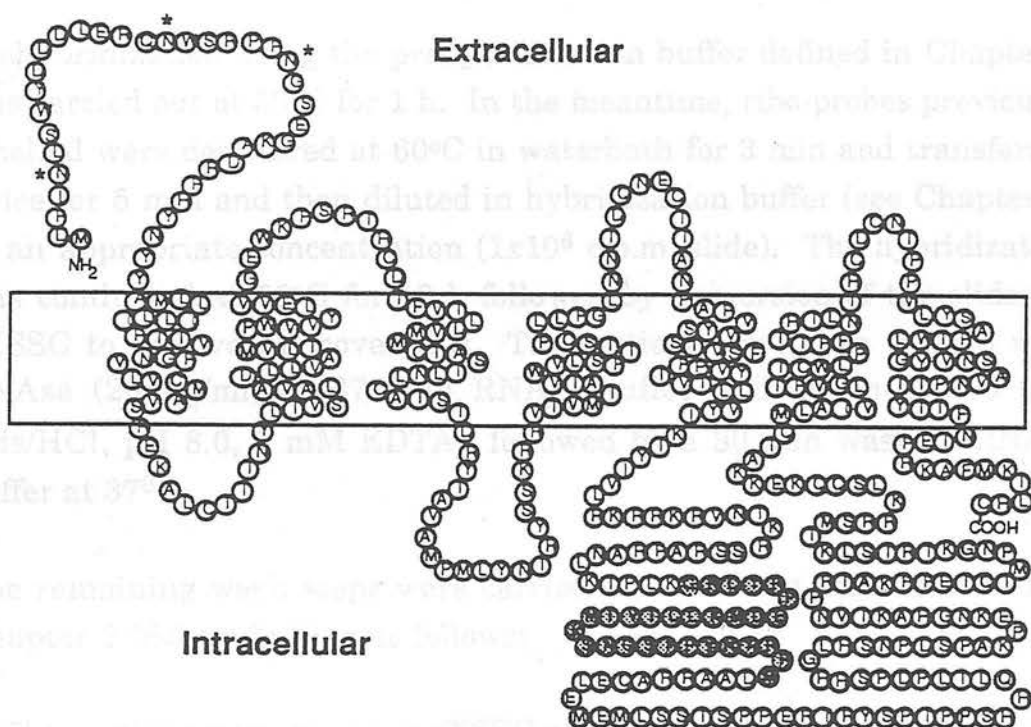


Fig. 7.1. Schematic representation of the predicted dopamine D2-B receptor structure encoded by the clone pZ19.12 cDNA. The amino acid sequence is shown in one-letter code and the protein is displayed in the seven-transmembrane domain configuration. The potential N-glycosylation sites in the amino-terminal extracellular domain are represented by asterisks. The shaded sequence shows the difference between dopamine D2 A and D2 B receptors.

fixed in freshly-made 4% paraformaldehyde for 5 min and washed twice in PBS for 10 min. The slides were then acetylated by washing briefly in 0.1 M triethanolamine/HCl buffer, pH8.0 (TEA buffer) and then immersed in 0.5% (v/v) acetic anhydride in TEA buffer for 20 min, followed by washing in 2XSSC twice for 10 min. All procedures were carried out at room temperature.

Prehybridization using the prehybridization buffer defined in Chapter 2 was carried out at 56°C for 1 h. In the meantime, ribo-probes previously labelled were denatured at 60°C in waterbath for 3 min and transferred to ice for 5 min and then diluted in hybridization buffer (see Chapter 2) at an appropriate concentration (1×10^6 c.p.m./slide). The hybridization was conducted at 56°C for 18 h followed by immersion of the slides in 4XSSC to remove the coverslips. The sections were then treated with RNase (20 µg/ml) at 37°C in RNase buffer (0.5 M NaCl, 100 mM Tris/HCl, pH 8.0, 1 mM EDTA), followed by a 30 min wash in RNase buffer at 37°C.

The remaining wash steps were carried out either as that described in Chapter 2 (Schedule 3) or as follows:

1. The sections were rinsed in 2XSSC at 60°C for 30 min;
2. Followed by 0.1XSSC at 60°C for 30 min;
3. Finally, washed with 0.1XSSC at RT for 3 min.

All the washes were carried out with 2 mM DTT in each dish except the last step and with gently stirring. The dehydration, autoradiography and development of autoradiography were performed as described previously in Chapter 2.

7.1.2.4. Northern blot analysis

RNA extraction and separation were undertaken as described in Chapter 2. However, different hybridization buffers and wash steps were examined as following:

A. Hybridization buffers

a. Buffer 1

<u>Stock Solution</u>	<u>Final concentration</u>
20XSSPE	5XSSPE
Formamide	5% (v/v)
100X Denhard's	5X
10% (w/v) SDS	0.5% (w/v)
10 mg/ml testis DNA	5 µg/ml

b. Buffer 2

<u>Stock Solution</u>	<u>Final concentration</u>
1M Sodium phosphate buffer	0.5M
0.5M EDTA (pH 8.0)	1 mM
BSA	1% (w/v)
10% SDS	7% (w/v)
Formamide	15% (v/v)

After BSA was dissolved in aqueous components, SDS and formamide were added to the solution.

Dopamine D2 receptor cDNA probes were constructed from a clone containing the full length of the dopamine D2 receptor (2.5 kilobase) and labelled with [32 P] by the random primer method (**Appendix 3**). After prehybridization overnight, the membrane with transferred RNA was hybridized for 24 h in 20 ml of hybridization buffer containing 1×10^6 c.p.m./ml alkali denatured [32 P] cDNA probes.

B. Washes

a. Wash step 1

1. The membrane was washed with 3XSSC with 0.1% (w/v) SDS at 65°C for 20 min;
2. Followed by 1XSSC with 0.1% (w/v) SDS at 65°C for 10 min.

b. Wash step 2

1. The membrane was briefly rinsed twice with 10 ml of prewarmed 2XSSC;
2. Then followed by washing with 30 ml of 2XSSC for 5, 10, and 30 min respectively at 65°C, renewed with fresh 2XSSC each time.

The development of film were carried out as described in Chapter 2, after exposing 1, 7 and 14 days.

7.1.2.5. Reverse transcriptase polymerase chain reaction (RT-PCR)

Term decidual cells showed the specific hybridization signals of dopamine D2 receptor mRNA by in situ hybridization, while this finding was not confirmed by Northern blot analysis. Therefore, PCR was used to cross-check whether decidual cells indeed contained dopamine D2 receptors.

A. Primers

Three pairs of rat and one pair of human dopamine D2 receptor primers were tested. The primers against the different regions of dopamine D2 receptor cDNA sequence were selected by the Genejock programme written by Dr. Phil Taylor, MRC Reproductive Biology Unit, for the Apple Macintosh computer and synthesized in 391 DNA Synthesizer (Applied Biosystems, PCR-MATE) and deprotected as presented in **Appendix 12**.

	Met	Asp	Pro	Leu	Asn	Leu	Ser	Trp	Tyr	Asp	Asp	Asp	Leu	Glu	Arg	Gln	Asn	17
1	ATG	GAT	CCA	CTG	AAC	CTG	TCC	TGG	TAC	GAT	GAC	GAT	CTG	GAG	AGG	CAG	AAC	
	Trp	Ser	Arg	Pro	Phe	Asn	Gly	Ser	Glu	Gly	Lys	Ala	Asp	Arg	Pro	His	Tyr	34
52	TGG	AGC	CGG	CCC	TTC	AAT	GGG	TCA	GAA	GGG	AAG	GCA	GAC	AGG	CCC	CAC	TAC	
	Asn	Tyr	Tyr	Ala	Met	Leu	Leu	Thr	Leu	Leu	Ile	Phe	Ile	Ile	Val	Phe	Gly	51
103	AAC	TAC	TAT	GCC	ATG	CTG	CTC	ACC	CTC	CTC	ATC	TTT	ATC	ATC	GTC	TTT	GGC	
	Asn	Val	Leu	Val	Cys	Met	Ala	Val	Ser	Arg	Glu	Lys	Ala	Leu	Gln	Thr	Thr	68
154	AAT	GTG	CTG	GTG	TGC	ATG	GCT	GTA	TCC	CGA	GAG	AAG	GCT	TTG	CAG	ACC	ACC	
	Thr	Asn	Tyr	Leu	Ile	Val	Ser	Leu	Ala	Val	Ala	Asp	Leu	Leu	Val	Ala	Thr	85
205	ACC	AAC	TAC	TTG	ATA	GTC	AGC	CTT	GCT	GTG	GCT	GAT	CTT	CTG	GTG	GCC	ACA	
	Leu	Val	Met	Pro	Trp	Val	Val	Tyr	Leu	Glu	Val	Val	Gly	Glu	Trp	Lys	Phe	102
256	CTG	GTA	ATG	CCG	TGG	GTT	GTC	TAC	CTG	GAG	GTG	GTG	<u>GGT GAG TGG AAA TTC</u>	(oligos, 13/8)				
	Ser	Arg	Ile	His	Cys	Asp	Ile	Phe	Val	Thr	Leu	Asp	Val	Met	Met	Cys	Thr	119
307	<u>AGC</u>	<u>AGG</u>	<u>ATT</u>	<u>CAC</u>	<u>TGT</u>	<u>GAC</u>	<u>ATC</u>	<u>TTT</u>	<u>GTC</u>	<u>ACT</u>	<u>CTG</u>	<u>GAT</u>	<u>GTC</u>	<u>ATG</u>	<u>ATG</u>	<u>TGC</u>	<u>ACA</u>	
	Ala	Ser	Ile	Leu	Asn	Leu	Cys	Ala	Ile	Ser	Ile	Asp	Arg	Tyr	Thr	Ala	Val	136
358	GCA	AGC	ATC	CTG	AAC	CTG	TGT	GCC	ATC	AGC	ATT	GAC	AGG	TAC	ACA	GCT	GTG	
	Ala	Met	Pro	Met	Leu	Tyr	Asn	Thr	Arg	Tyr	Ser	Ser	Lys	Arg	Arg	Val	Thr	153
409	GCA	ATG	CCC	ATG	CTG	TAT	AAC	ACA	CGC	TAC	AGC	TCC	AAG	CGC	CGA	GTT	ACT	
	Val	Met	Ile	Ala	Ile	Val	Trp	Val	Leu	Ser	Phe	Thr	Ile	Ser	Cys	Pro	Leu	170
460	GTC	ATG	ATT	GCC	ATT	GTC	TGG	GTC	CTG	TCC	TTC	ACC	ATC	TCC	<u>TGC CCA CTG</u>	(oligo, 14)		
	Leu	Phe	Gly	Leu	Asn	Asn	Thr	Asp	Gln	Asn	Glu	Cys	Ile	Ile	Ala	Asn	Pro	187
511	<u>CTC</u>	<u>TTC</u>	<u>GGA</u>	<u>CTC</u>	<u>AAC</u>	<u>AAT</u>	<u>ACA</u>	<u>GAC</u>	<u>CAG</u>	<u>AAT</u>	<u>GAG</u>	<u>TGT</u>	<u>ATC</u>	<u>ATT</u>	<u>GCC</u>	<u>AAC</u>	<u>CCT</u>	
	Ala	Phe	Val	Val	Tyr	Ser	Ser	Ile	Val	Ser	Phe	Tyr	Val	Pro	Phe	Ile	Val	204
562	GCC	TTT	GTG	GTC	TAC	TCC	TCC	ATT	GTC	TCA	TTC	TAC	GTG	CCC	TTC	ATC	GTC	
	Thr	Leu	Leu	Val	Tyr	Ile	Lys	Ile	Tyr	Ile	Val	Leu	Arg	Lys	Arg	Arg	Lys	221
613	ACT	CTG	CTG	GTC	TAT	ATC	AAA	ATC	TAC	ATC	GTC	CTC	CGG	AAG	CGC	CGG	AAG	
	Arg	Val	Asn	Thr	Lys	Arg	Ser	Ser	Arg	Ala	Phe	Arg	Ala	Asn	Leu	Lys	Thr	238
664	CGG	GTC	AAC	ACC	AAG	CGC	AGC	AGT	CGA	GCT	TTC	AGA	GCC	AAC	CTG	AAG	ACA	
	Pro	Leu	Lys	Gly	Asn	Cys	Thr	His	Pro	Glu	Asp	Met	Lys	Leu	Cys	Thr	Val	255
715	CCA	CTC	AAG	GGC	AAC	TGT	ACC	CAC	CCT	GAG	GAC	ATG	AAA	CTC	TGC	ACC	GTT	
	Ile	Met	Lys	Ser	Asn	Gly	Ser	Phe	Pro	Val	Asn	Arg	Arg	Arg	Met	Asp	Ala	272
766	ATC	ATG	AAG	TCT	AAT	GGG	AGT	TTC	CCA	GTG	AAC	AGG	CGG	AGA	ATG	GAT	GCT	
	Ala	Arg	Arg	Ala	Gln	Glu	Leu	Glu	Met	Glu	Met	Leu	Ser	Ser	Thr	Ser	Pro	289
817	GCC	CGC	CGA	GCT	CAG	GAG	CTG	GAA	ATG	GAG	ATG	CTG	TCA	AGC	ACC	AGT	CCC	
	Pro	Glu	Arg	Thr	Arg	Tyr	Ser	Pro	Ile	Pro	Pro	Ser	His	His	Gln	Leu	Thr	306
868	CCA	GAG	AGG	ACC	CGG	TAT	AGC	CCC	ATC	CCT	CCC	<u>AGT CAC CAC CAG CTC ACT</u>	(oligo, 1)					
	Leu	Pro	Asp	Pro	Ser	His	His	Gly	Leu	His	Ser	Asn	Pro	Asp	Ser	Pro	Ala	323
919	CTC	CCT	GAT	CCA	TCC	CAC	CAC	GGC	CTA	CAT	AGC	AAC	CCT	GAC	AGT	CCT	GCC	
	Lys	Pro	Glu	Lys	Asn	Gly	His	Ala	Lys	Ile	Val	Asn	Pro	Arg	Ile	Ala	Lys	340
970	AAA	CCA	GAG	AAG	AAT	GGG	CAC	GCC	AAG	ATT	GTC	AAT	CCC	AGG	ATT	GCC	AAG	
	Phe	Phe	Glu	Ile	Gln	Thr	Met	Pro	Asn	Gly	Lys	Thr	Arg	Thr	Ser	Leu	Lys	357
1021	TTC	TTT	GAG	ATC	CAG	ACC	ATG	CCC	AAT	GGC	AAA	ACC	CGG	ACC	TCC	CTT	AAG	
	Thr	Met	Ser	Arg	Arg	Lys	Leu	Ser	Gln	Gln	Lys	Glu	Lys	Lys	Ala	Thr	Gln	374
1072	ACG	ATG	AGC	CGC	AGA	AAG	CTC	TCC	CAG	CAG	AAG	GAG	AAG	AAA	GCC	ACT	CAG	
	Met	Leu	Ala	Ile	Val	Leu	Gly	Val	Phe	Ile	Ile	Cys	Trp	Leu	Pro	Phe	Phe	391
1123	ATG	CTT	GCC	ATT	GTT	CTC	GGT	GTG	TTC	ATC	ATC	TGC	TGG	CTG	CCC	TTC	TTC	
	Ile	Thr	His	Ile	Leu	Asn	Ile	His	Cys	Asp	Cys	Asn	Ile	Pro	Pro	Val	Leu	408
1174	ATC	ACG	CAC	ATC	CTG	AAT	ATA	CAC	TGT	GAT	TGC	AAC	ATC	CCA	CCA	GTC	CTC	
	Tyr	Ser	Ala	Phe	Thr	Trp	Leu	Gly	Tyr	Val	Asn	Ser	Ala	Val	Asn	Pro	Ile	425
1225	TAC	AGC	GCC	TTC	ACA	TGG	CTG	GGC	TAT	GTC	AAC	AGT	GCC	GTC	AAC	CCC	ATC	
	Ile	Tyr	Thr	Thr	Phe	Asn	Ile	Glu	Phe	Arg	Lys	Ala	Phe	Met	Lys	Ile	Leu	442
1276	ATC	TAC	ACC	ACC	TTC	AAC	ATC	GAG	TTC	CGC	AAG	GCC	TTC	ATG	AAG	ATC	TTG	
	His	Cys	Stop															444

Fig. 7.2. The amino acid and nucleotide sequences of rat dopamine D2-B receptor. The sequence of the different oligonucleotides used for PCR are shown by the underlined sequences. The inserted amino acid sequence (AA 242 to 270) of the dopamine D2-B receptor is shown in the *italic* regions

The sequences of the primers against rat and human dopamine D2 receptor cDNAs are shown in Fig. 7.3 and Table 7.3.

	Met	Asp	Pro	Leu	Asn	Leu	Ser	Trp	Tyr	Asp	Asp	Asp	Leu	Glu	Arg	Gln	Asn	17
29	ATG	GAT	CCA	CTG	AAT	CTG	TCC	TGG	TAT	GAT	GAT	GAT	CTG	GAG	AGG	CAG	AAC	
	Trp	Ser	Arg	Pro	Phe	Asn	Gly	Ser	Asp	Gly	Lys	Ala	Asp	Arg	Pro	His	Tyr	34
81	TGG	AGC	CGG	CCC	TTC	AAC	GGG	TCA	GAC	GGG	AAG	GCG	GAC	AGA	CCC	CAC	TAC	
	Asn	Tyr	Tyr	Ala	Thr	Leu	Leu	Thr	Leu	Leu	Ile	Ala	Val	Ile	Val	Phe	Gly	51
132	AAC	TAC	TAT	GCC	ACA	CTG	CTC	ACC	CTG	CTC	ATC	GCT	GTC	ATC	GTC	TTC	GGC	
	Asn	Val	Leu	Val	Cys	Met	Ala	Val	Ser	Arg	Glu	Lys	Ala	Leu	Gln	Thr	Thr	68
183	AAC	GTG	CTG	GTG	TGC	ATG	GCT	GTG	TCC	CGC	GAG	AAG	GCG	CTG	CAG	ACC	ACC	
	Thr	Asn	Tyr	Leu	Ile	Val	Ser	Leu	Ala	Val	Ala	Asp	Leu	Leu	Val	Ala	Thr	85
234	ACC	AAC	TAC	CTG	ATC	GTC	AGC	CTC	GCA	GTG	GCC	GAC	CTC	CTC	GTC	GCC	ACA	
	Leu	Val	Met	Pro	Trp	Val	Val	Tyr	Leu	Glu	Val	Val	Gly	Glu	Trp	Lys	Phe	102
285	CTG	GTC	ATG	CCC	TGG	GTT	GTC	TAC	CTG	GAG	GTG	GTA	GGT	GAG	<u>TGG AAA TTC</u>	(oligo, 8)		102
	Ser	Arg	Ile	His	Cys	Asp	Ile	Phe	Val	Thr	Leu	Asp	Val	Met	Met	Cys	Thr	119
336	<u>AGC AGG ATT CAC</u>	TGT	GAC	ATC	TTC	GTC	ACT	CTG	GAC	GTC	ATG	ATG	TGC	ACG				
	Ala	Ser	Ile	Leu	Asn	Leu	Cys	Ala	Ile	Ser	Ile	Asp	Arg	Tyr	Thr	Ala	Val	136
387	GCG	AGC	ATC	CTG	AAC	TTG	TGT	GCC	ATC	AGC	ATC	GAC	AGG	TAC	ACA	GCT	GTG	
	Ala	Met	Pro	Met	Leu	Tyr	Asn	Thr	Arg	Tyr	Ser	Ser	Lys	Arg	Arg	Val	Thr	153
438	GCC	ATG	CCC	ATG	CTG	TAC	AAT	ACG	CGC	TAC	AGC	TCC	AAG	CGC	CGG	GTC	ACC	
	Val	Met	Ile	Ser	Ile	Val	Trp	Val	Leu	Ser	Phe	Thr	Ile	Ser	Cys	Pro	Leu	170
489	GTC	ATG	ATC	TCC	ATC	GTC	TGG	GTC	CTG	TCC	TTC	ACC	ATC	TCC	TGC	CCA	CTC	
	Leu	Phe	Gly	Leu	Asn	Asn	Ala	Asp	Gln	Asn	Glu	Cys	Ile	Ile	Ala	Asn	Pro	187
540	CTC	TTC	GGA	CTC	AAT	AAC	GCA	GAC	CAG	AAC	GAG	TGC	ATC	ATT	GCC	AAC	CCG	
	Ala	Phe	Val	Val	Tyr	Ser	Ser	Ile	Val	Ser	Phe	Tyr	Val	Pro	Phe	Ile	Val	204
591	GCC	TTC	GTG	GTC	TAC	TCC	TCC	ATC	GTC	TCC	TTC	TAC	GTG	CCC	TTC	ATT	GTC	
	Thr	Leu	Leu	Val	Tyr	Ile	Lys	Ile	Tyr	Ile	Val	Leu	Arg	Arg	Arg	Arg	Lys	221
642	ACC	CTG	CTG	TAT	ATC	AAG	ATC	TAC	ATT	GTC	CTC	CGC	AGA	CGC	CGC	AAG		
	Arg	Val	Asn	Thr	Lys	Arg	Ser	Ser	Arg	Ala	Phe	Arg	Ala	His	Leu	Arg	Ala	238
693	CGA	GTC	AAC	ACC	AAA	CGC	AGC	AGC	CGA	GCT	TTC	AGG	GCC	CAC	CTG	AGG	GCT	
	Pro	Leu	Lys	Gly	Asn	Cys	Thr	His	Pro	Glu	Asp	Met	Lys	Leu	Cys	Thr	Val	255
744	CCA	CTA	AAG	GGC	AAC	TGT	ACT	CAC	CCC	GAG	GAC	ATG	AAA	CTC	TGC	ACC	GTT	
	Ile	Met	Lys	Ser	Asn	Gly	Ser	Phe	Pro	Val	Asn	Arg	Arg	Arg	Val	Glu	Ala	272
795	ATC	ATG	AAG	TCT	AAT	GGG	AGT	TTC	CCA	GTG	AAC	AGG	CGG	AGA	GTG	GAG	GCT	
	Ala	Arg	Arg	Ala	Gln	Glu	Leu	Glu	Met	Glu	Met	Leu	Ser	Ser	Thr	Ser	Pro	289
846	GCC	CGG	CGA	GCC	CAG	GAG	CTG	GAG	ATG	GAG	ATG	CTC	TCC	AGC	ACC	AGC	CCA	
	Pro	Glu	Arg	Thr	Arg	Tyr	Ser	Pro	Ile	Pro	Pro	Ser	His	His	Gln	Leu	Thr	306
897	CCC	GAG	AGG	ACC	CGG	TAC	AGC	CCC	ATC	CCA	CCC	AGC	<u>CAC CAC CAG</u>	CTG	ACT	(oligo, 1)		
	Leu	Pro	Asp	Pro	Ser	His	His	Gly	Leu	His	Ser	Thr	Pro	Asp	Ser	Pro	Ala	323
948	CTC	CCC	GAC	CCG	TCC	CAC	CAT	GGT	CTC	CAC	AGC	ACT	CCC	GAC	AGC	CCC	GCC	
	Lys	Pro	Glu	Lys	Asn	Gly	His	Ala	Lys	Asp	His	Pro	Lys	Ile	Ala	Lys	Ile	340
999	AAA	CCA	GAG	AAG	AAT	GGG	CAT	GCC	AAA	GAC	CAC	CCC	AAG	ATT	GCC	AAG	ATC	
	Phe	Glu	Ile	Gln	Thr	Met	Pro	Asn	Gly	Lys	Thr	Arg	Thr	Ser	Leu	Lys	Thr	357
1050	TTT	GAG	ATC	CAG	ACC	ATG	CCC	AAT	GGC	AAA	ACC	CGG	ACC	TCC	CTC	AAG	ACC	
	Met	Ser	Arg	Arg	Lys	Leu	Ser	Gln	Gln	Lys	Glu	Lys	Lys	Ala	Thr	Gln	Met	374
1101	ATG	AGC	CGT	AGG	AAG	CTC	TCC	CAG	CAG	AAG	GAG	AAG	AAA	GCC	ACT	CAG	ATG	
	Leu	Ala	Ile	Val	Leu	Gly	Val	Phe	Ile	Ile	Cys	Trp	Leu	Pro	Phe	Phe	Ile	391
1152	CTC	GCC	ATT	GTT	CTC	GGC	GTG	TTC	ATC	ATC	TGC	TGG	CTG	CCC	TTC	TTC	ATC	
	Thr	His	Ile	Leu	Asn	Ile	His	Cys	Asp	Cys	Asn	Ile	Pro	Pro	Val	Leu	Tyr	408
1203	ACA	CAC	ATC	CTG	AAC	ATA	CAC	TGT	GAC	TGC	AAC	ATC	CCG	CCT	GTC	CTG	TAC	
	Ser	Ala	Phe	Thr	Trp	Leu	Gly	Tyr	Val	Asn	Ser	Ala	Val	Asn	Pro	Ile	Ile	425
1254	AGC	GCC	TTC	ACG	TGG	CTG	GGC	TAT	GTC	AAC	AGC	GCC	GTG	AAC	CCC	ATC	ATC	
	Tyr	Thr	Thr	Phe	Asn	Ile	Glu	Phe	Arg	Lys	Ala	Phe	Leu	Lys	Ile	Leu	His	442
1305	TAC	ACC	ACC	TTC	AAC	ATT	GAG	TTC	CGC	AAG	GCC	TTC	CTG	AAG	ATC	CTC	CAC	
	cys stop																	443

Fig. 7.3. The amino acid and nucleotide sequences of human dopamine D2-B receptor. The sequence of the different oligonucleotides used for PCR are shown by the underlined sequences. The inserted amino acid sequence (AA 242 to 270) of the dopamine D2-B receptor is shown in the *italic* regions.

The sequences of the primers against rat and human pituitary dopamine D2 receptor cDNA are shown in Fig. 7.2. and Fig. 7.3.

B. RT-PCR protocol

Total RNA from early and term decidua was extracted as previously described. Rat pituitary poly-A- RNA, kindly supplied by Ms J. Zabavnik, MRC Reproductive Biology Unit, was used as a positive control.

All the reagents except the reverse transcriptase and RNase inhibitor were included in the GeneAmp DNA Amplification Reagent Kit and purchased from Perkin Elmer Cetus (Perkin Elmer Cetus, 761 Main Ave., Norwalk, CT 06859). AMV reverse transcriptase and RNase inhibitor were purchased from Promega Corporation (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711-5399, USA).

RT-PCR DNA amplification was carried out in 20 μ l reaction mixtures containing 3 μ g of decidua RNA (early or term pregnancy) or 1 μ g of rat pituitary poly-A-RNA, in 50 mM KCl, 10 mM Tris/HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.1% gelatin, each primer at 1.5 μ g, each dNTP (dATP, dCTP, dTTP, dGTP) at 10 mM, Ampli Taq polymerase at 9 units, RNase inhibitor at 15 units and 8 unit reverse transcriptase. The samples were then overlaid with 1 drop (40 μ l) of mineral oil to prevent condensation and subjected to 30 cycles of amplification. In two experiments, the products from the first 30 cycles of PCR were recycled with fresh reaction mixtures for additional 30 cycles to ensure adequate amplification.

After reverse transcription from RNA to DNA was completed at 42°C for 40 min, the samples were then heated to 94°C over 3 min to inactivate the reverse transcriptase and additional 1.5 min at 93°C to denature the double strand DNA. After cooling to 55°C over 1 min to anneal the primers with sample the sample was then heated to 72°C in 2 min to activate the polymerase and extend the annealed primers. Cycling was terminated after a final extension at 72°C for 7 min to

ensure that final extension was complete. Reaction was stopped by chilling to 4°C.

Thermal cycling was performed in a programmable Thermal Reactor (Hybaid, Hook & Tucker Instruments LTD, UK). Each amplified sample was checked on a 0.8% agarose gel stained with ethidium bromide and visualized on UV transilluminator.

7.1.3. Results

7.1.3.1. The effect of bromocriptine on decidual cells

The amount of PRL produced by the decidual cells treated with bromocriptine at 1×10^{-6} , 1×10^{-7} , 1×10^{-8} , and 1×10^{-9} M for 3 days were not statistically different from that secreted by control decidual cells (Fig.7.4.). Addition of oestradiol and progesterone did not alter the lack of effect of bromocriptine in decidual PRL production (Fig. 7.5.).

7.1.3.2. In situ hybridization

Radiolabelled antisense probes to dopamine D2 receptor gave the specific hybridization signals, albeit at low level, over the cytoplasm of the decidual cells (Plate 7.1. A). This pattern of hybridization was identical when different washing steps were tested. Furthermore, reproducible results were obtained in different tissues from 3 patients.

In contrast, sense dopamine D2 receptor probe radiolabelled at a similar specific activity and used under identical concentrations and conditions, showed only a few silver grains scattered over the decidual cells (Plate 7.1. B).

Specific hybridization was observed in the anterior and intermediate rat pituitary, when antisense probes were hybridized to rat pituitary. In the rat pituitary, the intermediate lobe displayed a high level of expression of dopamine D2 receptor and a relatively lower expression occurred in anterior pituitary lobe. However no silver grains above the level of non-specific binding were localized in posterior lobe (Plate

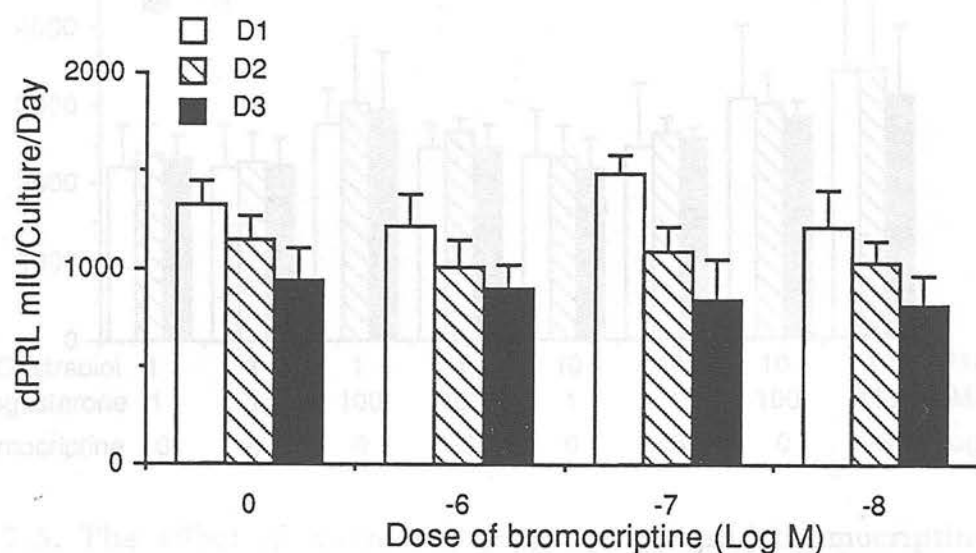


Fig. 7.4. The effect of bromocriptine on decidual PRL production from untransfected term decidual cells during a 3 day period of in vitro culture. Four replicate cultures of decidual cells from 4 different tissue were exposed to either the control medium or medium containing 10^{-6} to 10^{-8} M bromocriptine. The results are expressed as the mean \pm S.E.M. Bromocriptine at any concentration tested had no effect on decidual PRL production from term decidual cells.

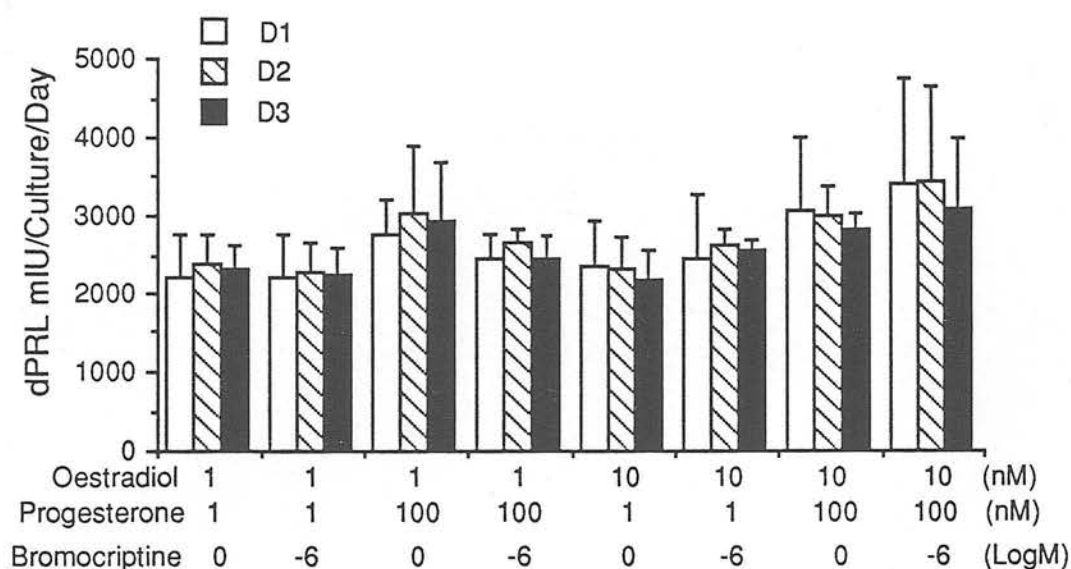


Fig. 7.5. The effect of oestradiol, progesterone and bromocriptine on decidual PRL production from term decidual cells during a 3 day in vitro culture observed in two patients. 1 or 10 nM oestradiol and 1 or 100 nM progesterone, alone or in combination, were added to the culture medium when the cells were plated. After attachment of the cells, the medium were changed and the cells were exposed to oestradiol and progesterone in presence or absence of bromocriptine (10^{-6} M). The presence of oestradiol and progesterone did not alter the lack of effect of bromocriptine on decidual PRL production.

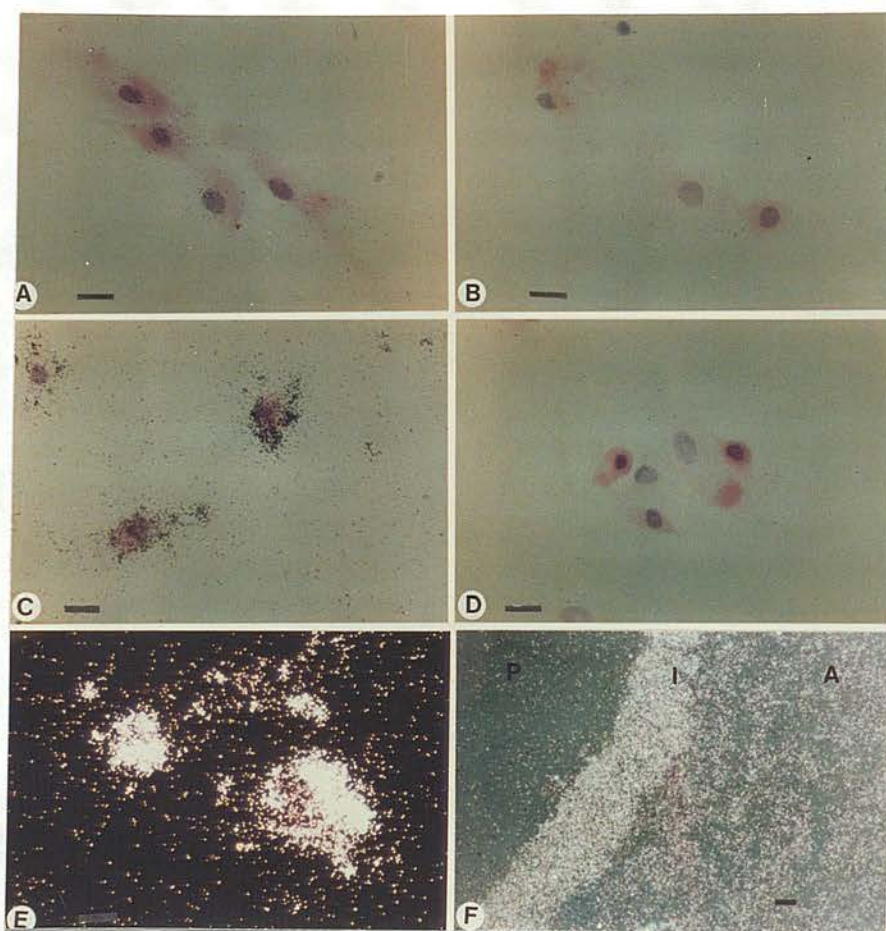


Plate 7.1. In situ localization of dopamine D2 receptor mRNA in untransfected and transfected human decidual cells. The scale bar is 20 μ m.

(7.1. A) Light field micrographs of untransfected human decidual cells after in situ hybridization with antisense riboprobe of dopamine D2 receptor showing the specific hybridization signals over the cytoplasm of the decidual cells.

(7.1. B) Light field micrographs of untransfected human decidual cells after in situ hybridization with sense riboprobe showing only non-specific labelling of the decidual cells.

(7.1. C) Light field micrographs of transfected human decidual cells after in situ hybridization with antisense riboprobe showing dramatically increased dopamine D2 receptor mRNA expressed in the transfected cells, which was diminished in the transfected decidual cells when hybridized to the sense probe (7.1.D).

(7.1.E) Dark field micrographs of transfected human decidual cells after in situ hybridization with antisense riboprobe shown dramatically increased dopamine D2 receptor mRNA expressed in the transfected decidual cells.

(7.1. F) Dark field photomicrographs of dopamine D2 receptor mRNA in rat pituitary after hybridization with the antisense riboprobe. There were intense hybridization signals in the intermediate lobe and specific labelling signals over the anterior lobe. The posterior lobe showed only non-specific background cDNA hybridization.

7.1.F). Sense probes showed only background hybridization signals in rat pituitary.

7.1.3.3. Northern blot analysis

No specific signal was observed on Northern blot analysis of human decidua mRNA from early or term decidua when using hybridization buffer 1 and wash buffer 1. Use of a low stringency hybridization buffer (buffer 2) and wash condition (step 2) also did not yield a specific signal. In contrast, a specific band of hybridization at 2.5 kb was observed on Northern blot analysis of sheep pituitary mRNA (Plate 7.2.) confirming that the probe and conditions were adequate to detect dopamine D2 receptor mRNA.

7.1.3.4. RT-PCR

One pair of rat primers (oligo 1 and 14) produced the amplified products with a molecular sizes of 400 bp, which could not be distinguished from degraded RNA on the 0.8% agarose gel (Plate 7.3.(1) from the top). A further two pairs of primers to rat dopamine D2 receptor (oligo 1 and 8, oligo 1 and 13) which gave an amplified products of about 600 bp were then used but no specific amplified product was identified from decidual mRNA although rat pituitary mRNA was amplified successfully (Plate 7.3.(2)). Both 5'primers exactly matched the human dopamine D2 receptor cDNA sequence, but there were mismatches of two nucleotides in 3'primer oligo (oligo 1) which might result in the failure of amplification of decidual mRNA.

In the final attempt to amplify decidual dopamine D2 receptor mRNA, a 3'primer corresponding exactly to the human pituitary dopamine D2 cDNA sequence was used. Again, no specific amplified product with the expected molecular size in human decidua was observed, while the specific amplified product was identified in the mRNA from rat pituitary (Plate 7.3.(3 and 4)).

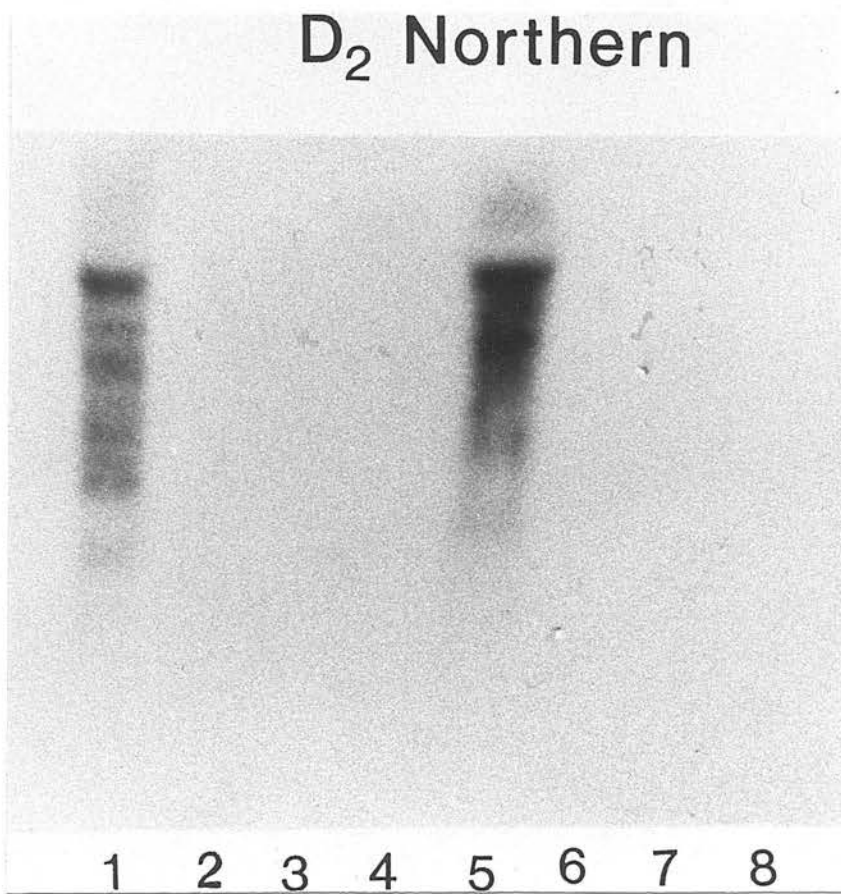
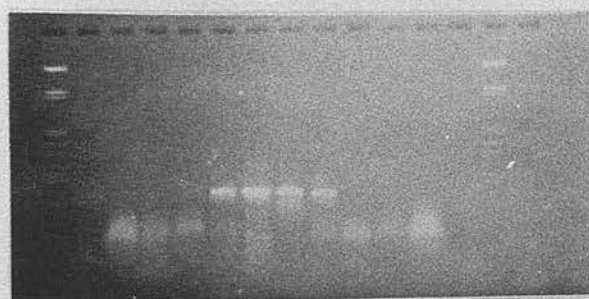


Plate 7.2. The autoradiographic detection of dopamine D₂ receptor mRNA in ovine and human early or term decidua by Northern blot analysis. The specific hybridization only occurred in the ovine pituitary (lane 1 and 5) and was absent in both early (lane 2 and 6) and term (lane 3, 4, 7 and 8) decidua. The total RNA loaded in the lanes 1 to 4 was 20 µg/lane and was 40 µg/lane in the lanes 5 to 8.

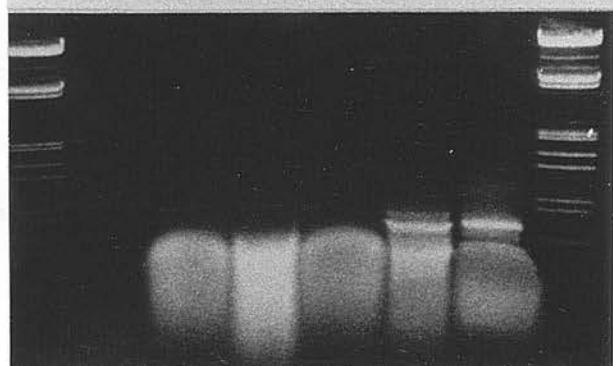
PCR of D₂ "R"



1 2 3 4 5 6



2 4 6 8 10 12 14



1 2 3 4 5 6 7 8



1 2 3 4 5 6 7 8

Plate 7.3. (The first photograph from the top) Reverse transcriptase PCR analysis of the dopamine D2 receptor mRNA in rat pituitary and human decidua. RT-PCR was performed on the poly-A RNA of rat pituitary or total RNA of human decidua using rat dopamine D2 receptor primers 1 and 14. Amplified products were run on a 0.8% agarose gel, stained with ethidium bromide and visualized on UV transilluminator. Note the amplified products (about 400bp) were not separated from the degraded primers and RNA.

Lane 1. no template (negative control); Lane 2 and 3. term pregnancy decidua; Lane 4. early pregnancy decidua; Lane 5. pZ19.12 cDNA clone (positive control). Lane 6. rat pituitary poly-A RNA (positive control).

Plate 7.3. (The second photograph) Reverse transcriptase PCR analysis of the dopamine D2 receptor mRNA in rat pituitary and human decidua performed under the same conditions as above., but using rat primers 1 and 8 (lane 2 to 7), and 1 and 13 (lane 8 to 13). Note that the amplified products were separated from the degraded RNA on the 0.8% agarose gel. No specific products were amplified from early or term decidua samples.

Lanes 1 and 14. DNA markers; Lanes 2 and 13. RNA prepared from RAG cells (negative control); Lanes 3, 5, 10 and 12. term pregnancy decidua; Lanes 4 and 11. early pregnancy decidua; Lanes 6 and 8. pZ19.12 cDNA clone (positive control). Lanes 7 and 9. rat pituitary poly-A RNA (positive control).

Plate 7.3. (The third and fourth photographs taken after running gel for 1.5 and 2.5 h, respectively) Reverse transcriptase PCR analysis of the dopamine D2 receptor mRNA in rat pituitary and human decidua performed under the same conditions as above, but using human dopamine D2 receptor primers 1 and 8. Note the specific amplified products were only observed in the lanes of rat pituitary samples. The human decidua from either early or term pregnancy had no specific bands shown on the gel.

Lanes 1 and 8. DNA markers; Lane 2. no template (negative control); Lanes 3 and 5. term pregnancy decidua; Lane 4. early pregnancy decidua; Lane 6. rat pituitary poly-A RNA (positive control); Lane 7. pZ19.12 cDNA clone (positive control).

7.1.4. Discussion

The present result is consistent with previous reports that the secretion of decidual PRL was not under dopamine control (Golander et al., 1979; Ben-Jonathan and Munsick, 1980). Furthermore, this study extends the previous study by showing that, even under appropriate environment of sex steroids, bromocriptine still did not affect PRL production by decidua. However, the mechanism of the failure of dopamine effect on decidual PRL production remains unclear. The present study is the first to examine the dopamine D2 receptor gene expression in human decidua, which is a prerequisite for dopamine action. Based on the theory that before the synthesis of a particular protein can begin, the corresponding mRNA molecules must be produced by DNA transcription, the expression of dopamine D2 receptor mRNA was evaluated in this study by in situ hybridization, Northern blot analysis and RT-PCR.

In situ hybridization was the first method employed in this study because of its great sensitivity and precise identification of the class of cells. It did indeed show that specific hybridization occurred in the cytoplasm of the decidual cells which had the characteristics of differentiated decidual cells, i.e. the size of the cells was four times bigger than that of other type of cells and contained enlarged nuclei. Moreover, the hybridization signals were reproducibly obtained from the tissue of the different patients and under variable hybridization and wash conditions. Furthermore, hybridization to sense probes showed only background staining. The stringency of the wash condition was adjusted by varying the salt concentrations in washing buffer. Poorly matched hybrids are only stable at high salt concentration, whereas in 0.1X SSC, only sequences which are well matched will remain. Additionally, posthybridization treatment with RNase will destroy any unhybridized probes. Using these conditions in the present study a signal indicating dopamine D2 receptor in decidual cells remained. Thus the result suggest the existence of dopamine D2 receptor in human decidua.

To double check the existence of dopamine D2 receptor in decidual cells Northern blot analysis of decidual mRNA was carried out. Under whatever hybridization conditions assessed in this study, no specific labelled band was observed. This technique requires homogenization of tissue and extraction of RNA, which might result in a loss of sensitivity if the target contained only a low level of the specific RNA to be detected (Shivers et al., 1986). With in situ hybridization, 10 to 100 molecules can be detected in a single cell, while the level of detection for Northern blot is about 10^5 to 10^6 target sequence molecules (Kawasaki, 1990). The negative results from Northern blot analysis therefore do not exclude the possibility of existence of dopamine D2 receptor gene expression in human decidua, albeit at a low level.

As a final attempt to determine whether the decidua indeed expressed dopamine D2 receptors, another powerful tool, RT-PCR, for detecting RNA molecules was utilized. As PCR is so sensitive, a single copy of the gene has been amplified (Innis and Gelfand, 1990). Another advantage for PCR is its specificity, in that the two oligoprimers complementary to the target DNA fragment are able to hybridize to opposite strands of the target sequence and are oriented so that the DNA synthesis by the polymerase proceeds across the region between the primers. In addition, the amplified products are also complementary to and capable of binding primers and each successive cycle doubles the amount of the specific DNA in the region between the two primers. The result is an exponential accumulation of the specific target fragment.

Under the RT-PCR conditions used in this study, again, no detectable dopamine D2 receptor amplified product could be visualized on the gel by using the primers either derived from the sequence of the rat or human dopamine D2 receptor. The system and primers were shown to amplify rat pituitary mRNA confirming that the failure to amplify decidual mRNA was not due to an inadequacy of the method.

It is therefore concluded that human decidua does not contain a dopamine D2 receptor identical to that in rat or human pituitaries. However, whether the decidua processes a similar structure to

Part II. Dopamine effect on decidual cells transfected with dopamine D2 receptor

7.2.1. Introduction

Since dopamine D2 receptor was not conclusively localized in the human decidual cells (Part I, Chapter 7), the lack of dopamine effect on decidual PRL production may be due to an absence of dopamine D2 receptor in human decidual cells. The next question is to ask if the dopamine D2 receptor is introduced into decidual cells, would they then response to dopamine? Therefore, the aim of the following study was to investigate the influence of bromocriptine on decidual cells transfected with dopamine D2 receptor gene.

7.2.2. Materials and methods

A previous study in the MRC Reproductive Biology Unit (Zabavnik et al., 1991) had successfully transfected a human kidney cell line with a rat dopamine D2 receptor construct using the Lipofectin, (N[1-(2,3-dioleyloxy)Propyl]-N,N,N-triethyl-ammonium) (DOMT) mediated transfection procedure. Therefore this procedure and construct were used in this study.

The transfection procedure was performed on human term decidual cells dispersed as described in Chapter 4. A full length dopamine D2 receptor cDNA clone kindly supplied by Dr. K. Eidne was used for transfection. The process of making purified DNA is detailed in **Appendix 9**.

Lipofecting reagent used as DNA delivery reagent was purchased from Bethes Research Laboratories (Bethes Research Laboratories, Life Technologies, Inc., Maryland, USA). The transfection procedure was accomplished according to the method description supplied by manufacture with slight modification as described in the following:

7.2.2.1. Formation of lipid-DNA complex

1 μ g DNA and 30 μ g Lipofectin for each well of 24-well plates were diluted separately to prevent precipitation in 250 μ l RPMI-1640 medium in polystyrene tubes and then combined and allowed to stand for 15 min at room temperature. RPMI-1640 medium used for transfection was serum-free to overcome the effect of any inhibitory component present in the serum.

7.2.2.2. Transfection procedure

Dispersed term decidual cells were plated in 24-well plates at a density 1×10^6 cells/well in 1 ml of RPMI-1640 medium supplemental with 10% fetal calf serum, 25 u/ml penicillin, 25 μ g/ml streptomycin, 50 μ g/ml gentamycin and 5 μ g/ml amphotericin B. The cells were then incubated in a humidified, 95% air:5% CO₂ environment at 37°C until they were 50-60% confluent. The decidual cells were then washed twice with 0.5 ml/well RPMI-1640 medium. After addition of 0.5 ml RPMI-1640 medium to each well, 0.5 ml lipid-DNA complex was dropwise and uniformly added to each well. For control wells, 250 μ l lipid with 750 μ l RPMI-1640 medium was introduced only. After incubation at 37°C in a humidified atmosphere of 95% air:5% CO₂ overnight, the medium was replaced with 1 ml of fresh RPMI-1640 medium supplemented with 20% fetal calf serum, with subsequent incubation for 1 day. The medium were then aspirated and the cells were treated with bromocriptine (1×10^{-6} , 1×10^{-7} , 1×10^{-8} , and 1×10^{-9} M) for an additional 24 h. The experiments were terminated by collecting the medium which, along with the medium from the cultured normal decidual cells was stored in -20°C prior to radioimmunoassay PRL as described previously. Tissues from 4 different patients were used in this study, and each treatment was run at 4 replicates.

Prior to the treatment of the cells with bromocriptine, two wells from either control or transfected cells were harvested by trypsinization for in situ hybridization to examine the dopamine D₂ receptor mRNA expression. After detachment from the wells, the cells were transferred to the chamber slides and incubated overnight for

reattachment of cells to the slides. The cultured cell slides were stored at -70°C after they were washed twice with PBS before processing for in situ hybridization.

7.2.2.3. Statistical analysis

The significance of the differences between different treatment was tested by analysis of variance. Contrasts between different groups were performed using Duncan's multiple range test.

7.2.3. Results

7.2.3.1. The expression of dopamine D2 receptor mRNA in decidual cell

A large concentration of dopamine D2 receptor mRNA was found in transfected decidual cells which indicated that the inserted clone of dopamine D2 gene was highly transcribed in the transfected decidual cells (Plate 7.1. C and E). No specific labelling signals were localized when the transfected decidual cells hybridized to sense probe (Plate 7.1. D). This result was reproducible in the decidual cells from the different patients.

7.2.3.2. The effect of bromocriptine on transfected decidual cells

Even after decidual cells expressed much higher level of dopamine D2 receptor mRNA than that of controls, bromocriptine at any concentrations tested had no effect on decidual PRL secretion 24 h of in vitro culture (Fig. 7.6.).

7.2.4. Discussion

Previous studies have determined beyond doubt that dopamine and its agonist, such as bromocriptine, were able to inhibit PRL secretion from the pituitary both in vivo and in vitro in all species tested (Macleod et al., 1974; Birge et al., 1970). Dopamine exerts its action through a membrane receptor, since specific dopaminergic receptor mRNA has

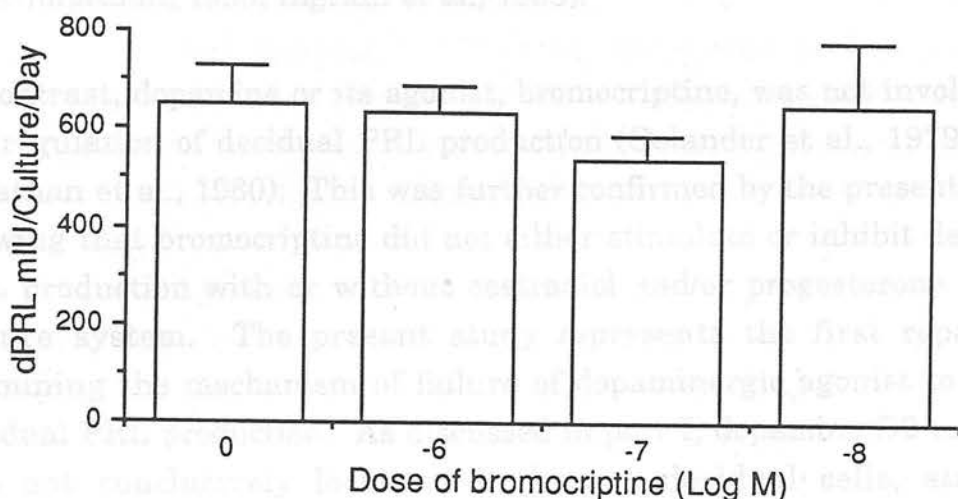


Fig. 7.6. The effect of bromocriptine on decidual PRL production of term decidual cells transfected with rat dopamine D2 receptor construct. Upon 1 day in vitro culture, there was no effect of bromocriptine at the concentrations 10^{-6} to 10^{-8} M on decidual PRL production from the transfected decidual cells.

However, some caution must be taken over these results. Signal transduction of the D2 receptor in the pituitary is mediated through G-proteins and at least two different G proteins, referred to as G_i and G_o , are involved (Snyder et al., 1987; Slater et al., 1989). Thus lack of the appropriate G-protein to couple with the transfected dopamine D2 receptor might account for the failure of bromocriptine to alter PRL production by transfected decidual cells. Again, G-proteins are not isolated directly from decidual and pituitary sources and the signalling pathways are not yet precisely ascertainable in these tissues.

been localized by in situ hybridization as well as PCR in both rat, human (Meador-woodruff et al., 1989; Toso et al., 1989) and sheep pituitaries (present study).

Dopaminergic signalling is transduced by two physiologically distinct plasma membrane receptors known D1 and D2 receptor (Kebabian and Calne, 1979). The D1 receptor was proposed to couple to adenylate cyclase and stimulate its activity, while D2 receptor had an inhibitory effect on this enzyme, and in turn decreased cAMP level inside cells to inhibit PRL production in pituitary, possibly by affecting K⁺ channels (Ben-Jonathan, 1985; Ingram et al., 1985).

In contrast, dopamine or its agonist, bromocriptine, was not involved in the regulation of decidual PRL production (Golander et al., 1979; Ben-Jonathan et al., 1980). This was further confirmed by the present study showing that bromocriptine did not either stimulate or inhibit decidual PRL production with or without oestradiol and/or progesterone in the culture system. The present study represents the first report on examining the mechanism of failure of dopaminergic agonist to affect decidual PRL production. As discussed in part I, dopamine D2 receptor was not conclusively localized in human decidual cells, strongly suggesting the conclusion that failure of dopaminergic agonist effect on decidual PRL production resulted from a lack of its receptor on decidual cells. However, even after dopamine D2 receptor was induced at a high level of expression in decidual cell by transfection with the dopamine receptor construct, bromocriptine still had no effect on decidual PRL production.

However, some caution must be taken over these results. Signal transduction of the D2 receptor in the pituitary is mediated through G-proteins and at least two different G proteins, referred to as G_i and G_o, are involved (Senogles et al., 1987; Elazar et al., 1989). Thus lack of the appropriate G protein to couple with the transfected dopamine D2 receptor might contribute to the failure of bromocriptine effect on PRL production by transfected decidual cells. Apart from G proteins, the second messenger system and machinery associated with cell signalling, are needed to produce measurable responses after the

ligand activation of the receptors. Moreover, it is uncertain at present time if the failure to show an inhibitory response to bromocriptine by transfected decidual cells is not due to the unstable transfection, since bromocriptine treatment on transfected decidual cells was only for 24 h. In some cases longer exposure time of pituitary cells to bromocriptine was needed before a measurable inhibitory response on PRL production could be observed (Ben-Jonathan et al., 1980), although an effect of bromocriptine on pituitary PRL production within 24 h is usually clearly observed. Since inhibition of pituitary PRL secretion by dopamine or its agonist was mediated through cAMP second message system (Ben-Jonathan, 1985) and cAMP was not involved in PRL production in the decidua (Chapter 6), there was no link between dopamine D2 receptor expressed in the transfected decidual cells and PRL regulation, which might result in the failure of bromocriptine to affect PRL production in transfected cells.

In conclusion: the present study, for the first time, revealed that the absence of dopaminergic inhibition of decidual PRL production might be due to the lack of dopamine receptors in decidual cells. However, no measurable response to bromocriptine by the decidual cells transfected with dopamine D2 receptor was observed. This may be due to the lack of appropriate G-protein associated with the D2 receptor.

cellular origin of decidual PRL was carried out in this thesis (Chapter 2), since it is extremely important in further determination of relevant regulators of decidual PRL.

Several groups have attempted to isolate these PRL-producing cells by a number of different methods. DeZiegler and Gurpide (1982) obtained an enriched PRL-producing cellular population from term decidua by enzymic digestion and selective attachment to plastic culture dishes. By employing isopycnic centrifugation, Markoff et al (1982a) has purified PRL-producing cells from term pregnancy. Similarly, Brown et al (1982) have isolated PRL-producing cells, confirmed by immunocytochemical staining of PRL, from early and mid pregnancy decidua. Results from their studies showed that PRL-producing cells were morphologically similar to decidual cells, but the cells were

CHAPTER 8

General Discussion

8.1. Identification of the cellular origin of decidual PRL

In early 1970's, large quantities of PRL were found in amniotic fluid through gestation (Tyson et al., 1972). Previous studies, either upon in vitro culture of pregnant and non-pregnant decidua (Maslar and Riddick, 1979; Maslar et al., 1980) or via identification of PRL mRNA in decidua by Northern blot (Clements et al., 1983) and in vitro translation (Taii et al., 1984), strongly suggested that the high concentration of PRL in amniotic fluid during human pregnancy was produced by decidua. This PRL appeared to be identical to pituitary PRL according to its chromatographic (Frame et al., 1979a), biological, immunological (Tomita et al., 1982a; 1982b) and binding properties (Golander et al., 1978). However, PRL secretion by decidua seems to be regulated quite differently from that in pituitary gland. Although the factors controlling PRL production have been extensively studied in pituitary tissue, much less is known about its regulation in decidual tissue. In order to investigate the control of decidual PRL production during human pregnancy, firstly, identification of the exact cellular origin of decidual PRL was carried out in this thesis (Chapter 2), since it is extremely important in further determination of relevant regulators of decidual PRL.

Several groups have attempted to isolate these PRL-producing cells by a number of different methods. DeZeigler and Gurpide (1982) obtained an enriched PRL-producing cellular population from term decidua by enzymic digestion and selective attachment to plastic culture dishes. By employing isopycnic centrifugation, Markoff et al (1982a) has purified PRL-producing cells from term pregnancy. Similarly, Braverman et al. (1984) have isolated PRL-producing cells, confirmed by immunocytochemical staining of PRL, from early and mid pregnancy decidua. Results from these studies showed that PRL-producing cells were morphologically similar to decidual cells, but the exact cell type

which was responsible for PRL production in decidua had not been conclusively localized.

While these studies suggested that only decidua in utero-placental unit was the source of PRL in amniotic fluid, PRL has also been localized by immunocytochemistry in the amnion (Healy et al., 1977), chorion (Frame et al., 1979b), trophoblast (Al-Timimi and Fox, 1986) and decidua from pregnant and non-pregnant women (Meuris et al., 1980). As immunocytochemistry can not distinguish between the site of synthesis and binding sites, it is not clear whether this PRL is synthesized in these PRL immunopositive cells or whether it is sequestered from the extra cellular space. In addition, the role of decidual PRL in utero-feto-placental unit is still obscure. Thus a positive identification of the site of synthesis of PRL together with a precise localization of the cell types which accumulate PRL should provide evidence for the potential sites of action of decidual PRL.

In situ hybridization enables the precise localization and identification of individual cells that contain the specific nucleic acid sequence. When used together with immunocytochemistry, both mRNA transcribed from the gene itself and the gene product translated from RNA can be co-localized in a particular cell type, which will give strong evidence for the site of synthesis. By developing an in situ hybridization method using a human pituitary PRL cDNA probe, together with immunocytochemistry to co-localize PRL mRNA and PRL in the studies reported in this thesis, decidual cells in the utero-placental unit were identified conclusively, for the first time, as the only source of PRL synthesis.

The use of a human pituitary PRL cDNA probe for in situ hybridization was validated by Northern blot analysis, showing that the probe hybridized to a single RNA species. It appeared that the decidual PRL mRNA was bigger than that of pituitary. This was confirmed by the recent reports that the decidual PRL mRNA is about 150 nucleotides longer than the human pituitary transcript attributed to differences in the 5' untranslated regulatory region of the gene (Gellersen et al.,

1989a; Hiraoka et al., 1991), further implying usage of a different transcription initiation site from that in the pituitary gene.

The results in Chapter 2 strongly suggest that only decidualized cells are capable of PRL biosynthesis. As PRL was also localized by immunocytochemistry in the amnion and placenta, but without co-localization of its mRNA, this finding indicated that cells of fetal origin, namely amnion and trophoblast, might be the potential target of PRL action. PRL receptors have also been identified in these tissue (McCoshen et al., 1982b; Healy et al., 1982; 1985; Herington et al., 1980), which is a mandatory prerequisite for any biological action of protein hormones. A number of studies have shown decidual PRL may be involved in maintaining amniotic fluid volume (Tyson et al., 1984), regulating fetal lung maturity (Hamosh and Hamosh, 1977; Grosso et al., 1980) and controlling parturition (Horrobin, 1973; Tyson et al., 1985).

This study was the first to identify decidual cells as the unique source of PRL in utero-placental unit by co-localization of PRL mRNA and its protein product using in situ hybridization and immunocytochemistry. The positive immunostaining in amnion and trophoblast indicated that decidual PRL play an important paracrine role within feto-placental unit.

8.2. The effect of oestrogen and progesterone on decidual PRL production

It is clear now that the high level of PRL in amniotic fluid is specifically produced by decidual cells. Decidual cells are differentiated from the stromal cells of the endometrium starting from luteal phase and continuing during pregnancy, mainly under the control of oestrogen and progesterone. In other words, the decidual cells are one of main targets of oestrogen and progesterone within uterus. Therefore, it would be logical to ask what is the potential role of oestrogen and progesterone on decidual PRL production.

Previous studies have suggested that the decidualization of the stromal cells in the endometrium is essential for the initiation of PRL

production (Maslar and Riddick, 1979; Daly et al., 1983a). Studies examining the potential effects of progesterone and oestrogen on PRL production of decidualized cells in normal menstrual cycle indicated that sex steroids, specially progesterone, were important regulators of decidual PRL production (Maslar et al., 1986). However, few studies have been performed on pregnant decidua, i.e. the control of oestrogen and progesterone on decidual PRL synthesis and release throughout pregnancy remains uncertain. Furthermore, many studies have not distinguished between regulation of de novo synthesis and secretion of PRL. Unlike pituitary, decidual PRL is not packaged into secretory granules (Handwerger et al., 1984), so it is important to examine the regulation of PRL at the level of synthesis.

Decidual cells continue to undergo further decidualization throughout pregnancy principally under the control of oestrogen and progesterone. To reveal the relationship between decidualization and PRL production, will give a clue as to how progesterone and oestrogen modify decidual PRL production during pregnancy. In Chapter 3 the relationship between the ability of the decidua to produce PRL at the different stages of pregnancy, the degree of decidualization of decidual cells in vivo, the PRL mRNA level determined by in situ hybridization and Northern blot, PRL content inside the decidual cells determined by immunocytochemistry, PRL output in amniotic fluid measured by radioimmunoassay was investigated. The results clearly showed that decidual PRL production is entirely dependent on decidualization in vivo (Chapter 3).

This study confirmed previous studies in the normal menstrual cycle that the capacity of the endometrium to produce PRL in vitro increased when decidualization spread throughout the endometrium (Maslar and Riddick, 1979; Maslar et al., 1980). However, the present results have extended these previous studies by demonstrating that decidualization, indicated by the enlargement of the cell size continued throughout the pregnancy, and had a close correlation with the PRL mRNA level. Thus, as decidualization progressed, more PRL gene was expressed in decidual cells, further confirmed by Northern blot analysis. Thus the ability of individual decidual cells to make PRL was magnified.

However, it is still not clear how decidualization activates decidual PRL gene expression. The results from immunocytochemistry to reveal gene product (PRL) contained inside decidual cells corresponded well with the result of in situ hybridization and Northern blot. As the decidualization spread over the decidua throughout pregnancy, the proportion of positive in situ and immunostaining cells in sections increased dramatically which was very obvious from early to mid pregnancy.

This is the first study using an in vivo model to examine decidual PRL regulatory mechanism at the level of both synthesis and secretion (Chapter 3). The results demonstrated that PRL gene expression in human decidual paralleled the degree of decidualization in vivo. Further study were carried out to examine the effect of the factors involved in decidualization on controlling PRL production in human decidua.

Progesterone is an essential factor for induction of decidualization, thus switching on decidual PRL production (Daly et al., 1983a) in the normal menstrual cycle. However, it is not clear how important the effects of progesterone on decidual PRL production are during early pregnancy. By blocking the action of progesterone with the antiprogestosterone RU 486 in vivo, the potential role of progesterone on PRL production was investigated in decidua parietalis, free of trophoblast, and decidua capsularis, with attached trophoblast (Chapter 4).

The results show for the first time that withdrawal of progesterone action in vivo resulted in suppression of decidual PRL production, with associated morphological de-decidualization in decidual parietalis. These results supported the concept that PRL production by decidual cells occurred as a consequence of decidualization which was induced by progesterone. However, no such effect occurred on decidua capsularis with trophoblast attached, which indicated that decidual PRL production was not only dependent on progesterone, but also potentially on factors derived from trophoblast (Handwerger et al., 1987a). In addition, secretion of PRL by decidual capsularis did not decline over the 5 days in in vitro culture in contrast to decidua

parietalis. This result implies that the trophoblast tissue attached to the capsularis may produce a factor(s), steroids or non-steroids, to maintain decidualization, and hence, PRL production, regardless of progesterone withdrawal.

In spite of the apparent dependence of PRL secretion on progesterone *in vivo*, it is of surprising that addition of progesterone *in vitro* could not stimulate PRL secretion (Chapter 4), confirming previous reports on PRL production by decidua *in vitro* from early human pregnancy (Bischof et al., 1986; Ren and Braunstein, 1990). It is well known that oestrogen priming is necessary for demonstration of progestational effect (Tamaya et al., 1985), and this may explain the absence of an effect of progesterone on decidual PRL secretion *in vitro* as oestradiol was not added to the cultured decidua. Thus the effect of progesterone may only be facilitative, maintaining decidual differentiation and PRL is only associated with the presence of progesterone-dependent differentiated decidual cells.

Decidualization in the human, in contrast to animals, is a spontaneous phenomena that occurs under the control of ovarian steroid hormones (Dallenbach-Hellweg, 1988). During pregnancy, syncytio-trophoblast cells synthesize not only progesterone but also oestrogen. To determine whether both sex steroids are required for the modulation of PRL release, the pattern of PRL produced by term decidual cells *in vitro* treated with or without progesterone and oestrogen, either alone or in combination, was investigated. The results demonstrated that PRL production was stimulated and maintained by the treatment of decidual cells with a combination of oestradiol and progesterone. Either progesterone or oestradiol alone *in vitro* was incapable of stimulating and maintaining PRL secretion from decidua. These results, again, suggest that progesterone is not a direct regulator of decidual PRL production and, further, support the hypothesis that, through decidualization, in which oestrogen is required, progesterone has an indirect effect on decidual PRL production.

The enhancement of progesterone-stimulated function by concurrent oestradiol treatment observed in this study is comparable with the

known synergistic action of oestrogen and progesterone on decidualization of the endometrium (Irwin et al., 1989). This is presumably related to the known up-regulation of progesterone receptors by oestrogen both in vivo (Lubbert et al., 1982) and in vitro (Eckert and Katzenellenbogen, 1981). Moreover, oestrogen is clearly the major stimulator of cell growth and proliferation of stromal cells (Pavik and Katzenellenbogen, 1978), which is a direct prerequisite for decidualization (Bell, 1983). Thus, oestrogen might be indirectly involved in the control of decidualization and, together with progesterone, act synergistically to maintain decidualization and the consequent PRL production by decidualized cells which was further confirmed by studies of the immunolocalization of oestradiol and progesterone receptors (Chapter 4 and 5).

It has been well established that steroid hormones can only act after binding to their specific receptors in the target (Gorski and Gannon, 1976). Decidual steroid receptors must therefore play a vital role in mediation of the decidual response to steroids. However, at present, no information is available to define the exact receptor status of decidua in relation to PRL production during pregnancy. As immunocytochemistry allows direct localization of steroid receptors and an evaluation of the receptors within a complex association of cell types in decidua parietalis and capsularis, the steroid receptor status in different regions of early pregnancy decidua, intracellular steroid receptor distribution and steroid receptor content throughout pregnancy in relation to PRL production were examined (Chapter 5).

The results clearly show that most decidual cells in either decidua capsularis or parietalis contain progesterone receptor. This confirmed a previous study that early pregnant decidua was characterized by a large concentration of progesterone receptors determined by binding assay (Levy et al., 1980) and provides direct evidence that both decidua parietalis and capsularis are under the control of progesterone. This corresponded to its main role in decidual cells to support their further growth and differentiation during pregnancy. These results give more direct evidence that failure of RU 486 to cause de-decidualization in decidua capsularis is not related to the alteration of the progesterone

receptor, but due to the factors derived from trophoblast which attached to decidua capsularis to maintain decidualization.

In contrast to the progesterone receptor, oestrogen receptor in decidua parietalis were mainly localized in glandular epithelia, while few decidual cells contained oestrogen receptor which may result from the down-regulation of oestrogen receptor by high local concentration of progesterone, as the decidual cells are a main target for progesterone during pregnancy. However, it remains to be shown whether the high level of oestrogen receptor in glandular cells contributes to a biological effect.

More intense staining of oestrogen and progesterone receptors was found in the decidual cells of decidua capsularis. This may be due to the high local concentration of oestrogen produced by the attached trophoblast which could up-regulate its own receptor and the progesterone receptor (Keitmann-Gimgal et al., 1979; Kreitmann-Gimgal et al., 1980; West et al., 1987). This would, therefore, enhance progesterone action in decidua capsularis to maintain PRL production as observed in Chapter 4.

Thus the difference between decidua capsularis and parietalis in producing PRL upon in vitro culture might result from oestrogen or other factors together with progesterone produced by attached trophoblast which participated in modulation of PRL production. This view was further confirmed in the study of term decidual cells in culture in which oestrogen in combination with progesterone modified PRL production (Chapter 4).

A striking observation made in this study was that in term decidual cells, progesterone receptor was localized in the cytoplasm, but not in nuclei. As steroid receptor immunocytochemistry was performed on frozen sections and under the same conditions as that of early pregnancy decidual staining, it is unlikely this cytoplasm staining was due to any artifacts. Recently, a new view of the mechanism of steroid action has been proposed based on the immunocytochemistry: both unoccupied and steroid-occupied receptors reside in nuclei even under

the environment of low steroid concentration (Welshon et al., 1984). Thus, whether this discrepancy in the intracellular localization of progesterone receptor in decidual cells resulted from localization of dissimilar forms of steroid receptors remained to be identified. Nevertheless, this cytoplasmic form of progesterone receptor appeared to be functional since progesterone combined with oestrogen could stimulate PRL production in term decidual cells (Chapter 4).

Combining the previous studies from other groups and the serial investigations of steroids and their receptors in this thesis (Chapter 2,3,4 and 5), several assumptions can be drawn: progesterone was required for induction and maintenance of decidualization in the human endometrium, thus switching on PRL production (Daly et al., 1983a; Maslar et al., 1986) in the normal menstrual cycle. This extended to pregnancy, since PRL gene expression was increased as decidualization progressed in pregnancy (Chapter 3). Moreover, the results of co-localization of progesterone receptor and PRL by double immunostaining (Chapter 5) further supports the concept discussed above, that PRL was only associated with the cells which contained progesterone receptors. Additionally It was shown that progesterone was only capable of inducing and maintaining decidualization in oestrogen-primed tissue since progesterone could modulate PRL production only when used together with oestrogen. As progesterone receptors are oestrogen-dependent, there may be an indirect association between oestrogen and progesterone at receptor level. This mechanism may be involved in controlling PRL production, with the combination of oestrogen and progesterone produced by trophoblast in decidua capsularis or added in culture medium, maintaining progesterone receptors and modulating PRL production.

8.3. The effect of non-steroid factors and dopamine on decidual PRL production

It was suggested that in normal pregnancy blastocyst-induced decidualization probably augments that initiated by sex steroids (Fin, 1977). This was further confirmed in studies in this thesis where as gestation advanced, decidualization progressed throughout pregnancy

(Chapter 3) with increasing expression of PRL mRNA in fully decidualized cells. In Chapter 4, it has been demonstrated that a factor(s) from trophoblast in decidua capsularis could maintain decidualization, with consequent maintenance of PRL production. The potential regulatory effect associated with placenta was then examined. The results in Chapter 6 support the concept that the placenta may be important in regulation of decidual PRL secretion, since significantly more PRL was released by term decidua exposed to placental conditioned medium, consistent with previous studies (Handwerger et al., 1983). However, the present study extends previous studies by demonstrating that neither acute nor chronic PRL-releasing activity was observed in early pregnancy incubated with early placental conditioned medium for either 1 h or 24 h, which indicated PRL releasing factor(s) was not involving in regulation of PRL production in early pregnancy.

Human chorionic gonadotrophin (hCG), a major protein from placenta, tested within physiological doses, failed to induce either an acute or a chronic response of decidual PRL production. This result, in agreement with other studies (Handwerger et al., 1983; Ying et al., 1988), did not support any role of hCG in regulation PRL production during pregnancy.

Since a number of physiological inhibitors and stimulators of pituitary PRL synthesis and release, such as TRH and dopamine, failed to affect PRL secretion from decidual cells, the second messenger system in decidua was investigated to determine whether the same intracellular signalling system was involved in controlling PRL production in both tissues. The inhibition of PRL secretion from pituitary lactotrophs by dopamine is linked to adenylate cyclase. The activity of this enzyme is reduced by the interaction of dopamine with D2 receptors, resulting in a decreased level cAMP (Ben-Jonathan, 1985). The stimulatory effect of TRH on PRL secretion and synthesis mediated through protein kinase C activation (Sutton and Martin, 1982). These two intracellular signalling factors, cAMP and protein kinase C, were chosen to be examined in this study (Chapter 6).

The current finding demonstrated that dibutyryl cAMP, an analogue of cAMP, in concentrations between 0.1 to 5 mM had no effect on decidual PRL production in either 0.5 h or 1 h exposure. However, activation of protein kinase C, achieved by TPA acting as functional analogue of diacylglycerol, competing for same receptor on the enzyme (Sharkey et al., 1984), inhibited decidual PRL production significantly in a time and dose dependent manner. This occurred even in the presence of oestrogen and progesterone indicating that the effect of TPA was to negate the positive effect of oestrogen and progesterone. This represents the first report of a long-term action of TPA on decidual PRL production. Gellersen et al. (1989) has reported a similar effect of TPA on reducing PRL mRNA synthesis in human IM-9-P3 cell line, which shares characteristics of decidual cells, with regard to its very small intracellular PRL pool, their unresponsiveness to many secretagogues controlling PRL secretion, and probably similar PRL gene structure in 5' untranslated region (DiMattia et al., 1990; Hiraoka et al., 1991).

The results in Chapter 6 support the concept that a local regulatory mechanism for decidual PRL production might exist, which is related to the placenta, but not hCG. The regulation of PRL secretion from decidua is distinct from that of pituitary PRL, even at the second messenger level. The adenylase system (dbcAMP) failed to affect, while TPA inhibited PRL production by term decidual cells.

In the pituitary, dopamine, a major physiological inhibitor of PRL production, acts through the dopamine D2 receptor. However, it not clear wether the failure of dopamine to affect PRL production in decidua occurs after binding to its receptor or because no dopamine receptor is present on the decidual cells. In the studies reported in Chapter 7, in situ hybridization, Northern blot and PCR were employed to investigate whether or not dopamine D2 receptor is present or absent on decidual cells. Although in situ hybridization appeared to indicate the presence of dopamine D2 receptors, albeit at a low level, on decidual cells, Northern blot and PCR of decidual mRNA with primers to both the rat and human dopamine D2 receptor sequence failed to confirm the presence of dopamine D2 receptors.

Thus, human decidual cells do not appear to contain a dopamine D2 receptor identical to that in rat or human pituitary. However, whether the decidual cells process a structure similar to the dopamine D2 receptor or the different subtypes of dopamine D2 receptor, as suggested by in situ hybridization, needs further investigation.

Since dopamine D2 receptor was not conclusively localized in human decidual cells, the lack of dopamine effect on decidual PRL production, as shown by previous studies (Golander et al., 1979; Ben-Jonathan et al., 1980) and confirmed in this study (Chapter 7), may be due to the absence of dopamine D2 receptor in human decidual cells. Then the next step (Chapter 7) was performed to investigate whether the introduction of dopamine D2 receptor into decidual cells would induce decidual cells to respond to bromocriptine. However, even after the dopamine receptor was introduced and expressed at a high level of its mRNA in decidual cells by transfection with a dopamine receptor construct, bromocriptine still had no effect on decidual PRL production. No measurable response to bromocriptine by decidual cells transfected with dopamine D2 receptor suggests that the machinery associated with cell signalling, such as relevant G-protein, second messenger system, is not functional.

8.4. The comparison of the regulatory systems involved in the synthesis and release of decidua and pituitary PRL

The marked difference of the regulatory systems involved in decidua and pituitary PRL synthesis and release have been demonstrated in previous (Golander et al., 1979; Handwerger et al., 1983; Rosenberg Bhatnagar, 1984) and present studies, the mechanism of which, however, remains poorly understood. Unlike pituitary gland, little hormone is stored in the decidual cells (Golander et al., 1978; Markoff et al., 1983a), further confirmed in this study (Chapter 6). In addition, decidual PRL is non-specifically bound to cellular components, while pituitary PRL is packaged into secretory granules (Handwerger et al., 1984). Furthermore, no chromogranin, a major protein component of secretory vesicles, was localized in the decidual cells by immunocytochemical techniques, in contrast again to the strong

positive staining in pituitary cells (Handwerger and Capel, 1985). These results suggest important differences in the peptide processing and secretory system in two tissues.

Decidual cells do not possess the cell surface ganglioside A2B5 and 3G5, which are all features shared by anterior pituitary and other endocrine cells that synthesis and release protein hormone (Handwerger and Capel, 1985). The decidual cells thus appear to represent a unique protein hormone-secreting cells. Furthermore, the lack of the respective receptors, such as dopamine D2 receptor as demonstrated in Chapter 7 or different intracellular cell signalling systems might also be attributed to this regulatory difference in these two tissue.

However, the main difference, as revealed recently, resides in the different gene regulatory region between decidua and pituitary PRL. In contrast to the PRL protein coding sequence in decidua identical to that in pituitary, as evidenced by in vitro translation system (Takahashi et al. 1984), recent studies have revealed that decidual PRL mRNA was approximately 150 nucleotides longer than the transcript from pituitary (Gellersen et al., 1989a; Chapter 2), which might result from the elongation of the 5'untranslated region of the PRL gene. It has been suggested that PRL gene in decidua contained a placenta-specific promoter and decidual PRL mRNA was encoded by six exons including a placenta-specific exon (DiMattia et al., 1990; Hiraoka et al., 1991). In addition, Pit-1, interacting with *cis*-acting elements located in the PRL gene of pituitary to direct the pituitary-specific transcription of the PRL gene (Davis, 1990), is not expressed in the human decidua (DiMattia et al., 1990; Brook, Davis and McNeilly, unpublished observation, 1991). Thus, the mechanism of the different regulators controlling the synthesis and secretion of PRL from pituitary and decidua resided in the different regulatory structure of the PRL gene, because *cis*-acting elements were responsible for transducing regulatory signals from the cell membrane to the pituitary PRL gene located in the immediate vicinity of the transcription initiation site associated with Pit-1 response elements (Elsholtz et al., 1986; Jackson and Bancroft, 1988).

Conclusion: The results reported in this thesis support the concept that decidual cells are the only source of PRL in utero-placental unit. The major control of decidual PRL release is the rate of PRL synthesis and this is related to the degree of decidualization, induced and maintained by a synergistic action of oestradiol and progesterone via the respective receptors throughout pregnancy. Dopamine is not a controller of decidual PRL production.

6. The slides were then immersed in poly-L-lysine (0.1 µg/ml) solution for 10 min. After drying, the slides were stored in black boxes at 4°C until use.

Appendix 2. In situ hybridization solution

A. Solutions

1. DEPC treated water
500 µl of 10 % (w/v) DEPC in absolute alcohol were added in 500 ml bottle by N₂ then 500 ml distilled water were added to a final concentration of 0.1% (w/v) DEPC, allowed to stand for 12 h, and then autoclaved. All the following solutions were made up with 0.1% (w/v) DEPC treated water.

2. Phosphate buffered saline (PBS), pH 7.2 (100 mM).
The following were added to 800 ml of DEPC treated water:
85.6g NaCl (1.3M)
20.7g Na₂HPO₄ (70mM)
3.9g NaH₂PO₄ (30mM)
Made up to 1 litre with DEPC treated water

3. 4% paraformaldehyde
4g of paraformaldehyde were dissolved in 100 ml of 10 mM PBS by heating to 60°C with stirring, cooled and filtered. This solution was made up fresh every day.

Appendix 1. Preparation of poly-L-lysine coated slides

Washed slides were further cleaned by the following procedures:

1. 95% ethanol for 5 min;
2. 0.5 M HCl in 95% ethanol for 5 min;
3. 95% ethanol twice, for 5 min;
4. The slides were rinsed with distilled water;
5. Baked at 250°C for 6 h;
6. The slides were then immersed in poly-L-lysine (0.1 µg/ml) solution for 10 min. After air-drying, the slides were stored in black boxes at 4°C until use.

Appendix 2. In situ hybridization solution

A. Solutions

1. DEPC treated water
500 µl of 10 % (v/v) DEPC in absolute alcohol were dried in 500 ml bottle by N₂, then 500 ml distilled water were added to a final concentration of 0.1% (v/v) DEPC, allowed to stand for 12 h, and then autoclaved. All the following solutions were made up with 0.1% (v/v) DEPC treated water.

2. Phosphate buffered saline (PBS), pH 7.2 (100 mM),

The followings were added to 800 ml of DEPC treated water

85.0g	NaCl	(1.3M)
10.7g	Na ₂ HPO ₄	(70mM)
3.9g	NaH ₂ PO ₄	(30mM)

Made up to 1 litre with DEPC treated water

3. 4% paraformaldehyde

4g of paraformaldehyde were dissolved in 100 ml of 10 mM PBS by heating to 60°C with stirring, cooled and filtered. This solution was made up fresh each day.

4. 20X standard saline citrate (20XSSC)

175.3g of NaCl and 88.2g of sodium citrate were dissolved in 800 ml of DEPC treated water, adjusted to pH 7.0 with 1N HCl, and made up to 1L, then autoclaved.

5. 0.2% Glycine

200 mg of glycine were dissolved in 100 ml of 10 mM PBS, this solution was made up fresh each day.

6. Proteinase K

The proteinase K was dissolved in DEPC treated water at a stock concentration of 10 mg/ml and stored at -20°C.

7. 0.1M Tris/HCl

Tris base 12.1g

DEPC treated water 800 ml

Adjusted to pH 7.5 by concentrated HCl. Made the volume of the solution up to 1L.

8. 1N HCl: made with DEPC treated water and autoclaved.

9. 50 mM CaCl₂: made with DEPC treated water and autoclaved.

10. RNase buffer: 20 mM Tris/HCl pH 7.6 containing 1 mM EDTA.

B. Hybridization buffer

1. Deionised formamide

50 ml of formamide were mixed with 5g of mixed-bed ion exchange resin (Bio-Rad AG 501-x8, 20-50 mesh). The solution was stirred for overnight at 4°C, filtered twice with whatman No.1 filter paper and stored at -20°C.

2. Denhart's solution (50X)

To each vial which contained 50 mg each of BSA, Ficoll, and PVP, 5 ml of DEPC treated water was added. The solutions was then aliquoted and stored at -20°C.

3. Salmon testis denatured DNA (10 mg/ml)
4. transfer RNA (10 mg/ml)
20 mg of tRNA was dissolved in 2 ml of DEPC treated water and placed on ice for 30 min, then aliquoted to storage volume and stored at -70°C.
5. 50% (w/v) dextran sulphate
1g of dextran sulphate was dissolved in 2 ml of DEPC treated water by heating to 37°C.
6. 10% (w/v) SDS: made with DEPC treated water.
7. 1M DTT
5g DTT was added to 32.4 ml DEPC treated water.
8. 25X SSPE
The following reagents were added to 80 ml DEPC treated water:
NaCl 26.3g
NaH₂PO₄ 2.98g
The pH was adjusted to 7.4, then 5 ml of 0.5M EDTA added. The solution was made up to 100 ml using DEPC treated water and then autoclaved.
1X SSPE being: 0.18 M NaCl, 10 mM NaH₂PO₄, pH 7.4 and 1 mM EDTA.

C. Hybridization buffer formula

Stock concentration Final concentration

100%	deionized formamide	50%
25X	SSPE	5X
50X (1% w/v)	denhart's	0.1%
50% (w/v)	dextran sulphate	4%
10 mg/ml	tRNA	200 µg/ml
10 mg/ml	stDNA	200 µg/ml
10% (w/v)	SDS	0.1%
1 M	DTT	10 mM

Prehybridization buffer was made up using DEPC treated water instead of dextran sulphate because it made the buffer too viscous and consequently was difficult to remove prior to application of the hybridization buffer.

Appendix 3. cDNA probe labelling by using Random Primer labelling method

cDNA probe was labelled with [^{35}S] dCTP for in situ hybridization and with α [^{32}P] dCTP for Northern blot analysis by the method of Feinberg and Vogelstein (1983) using a random primed DNA labelling kit (Boehringer Mannheim, Lewes, Sussex, UK).

1. 20 ng hPRL DNA in 10 mM Tris/HCl buffer was aliquoted to a new eppendorf tube and boiled for 10 min to denature the DNA;

2. The following reagents were added in order:

1 μl of each dNTP (dTTP, dTTP, dATP)

2 μl of reaction buffer

5 μl [^{35}S] dCTP or α [^{32}P] dCTP

1 μl Klenow

Incubated for 1 hour at 37°C in dryblock in hot lab.

3. Labelled DNA was separated on a NICK Column (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) by eluting with three times 400 μl of equilibration buffer (10 mM Tris/HCl and 1 mM EDTA).

6. Samples were collected into three tubes.

7. 2 μl from each tube was counted directly on a scintillation counter to assess specific activity of the probe in counts per min (cpm).

Appendix 4. Autoradiography

A. Photographic emulsion

1. Two vials of Kodak emulsion, which was stored at 4°C, were warmed at 45°C for 45 min.

2. Under the safe light in dark room, the melt emulsion was poured into a dipping chamber and set back in the 45°C waterbath.

3. Some cleaned blank slides were dipped in to remove bubbles.
4. Each slide was dipped and withdrew slowly from the emulsion once. The bottom edge of the slides was blot on a stack of paper towel. The slides were kept vertical, and allowed to stand up in a test tube rack to dry for 30 min after the last slide was dipped.
5. The slides were transferred to a light-tight box for 45 min before putting into the black plastic slide boxes containing a tube of drier.
6. The slides were allowed to expose at 4°C for 7 to 14 days.

B. Developing the emulsion coated slides

1. The following solutions were cooled on ice to balance the temperature to 15°C.

1. 350ml developer (Kodak developer);
2. 400 ml distilled water (Stopper);
3. 80 ml fix plus 320 ml distilled water (fix);
4. 400 ml distilled water.

2. Under safe light, the slides were taken through:

1. developer for 4 min;
2. stopper for 20 seconds;
3. fix for 10 min;
4. water for 15 min

3. The slides were then rinsed in running tap water for at least 15 min. Light was on at this point.

4. The slides were stained with hemotoxylin and eosin within a couple of hours.

Appendix 5. Preparation of immunocytochemical solutions

1. 4% neutral buffered formalin was made up with the following reagents:

- a) 100 ml of formalin (40% w/v formaldehyde);
- b) 4g of sodium phosphate (monobasic and monohydrate);
- c) 6.5g of sodium phosphate (dibasic and anhydrous);

The solution was made up to 1L with distilled water.

2. 4-Chloro-Naphthol-sbstrate solution

3 mg of 4-Chloro-Naphthol (Sigma) was dissolved in 0.1 ml of absolute alcohol with stirring, which was then added to 10 ml of 0.05 M Tris buffer, pH 7.6 with 0.1% H₂O₂. The solution was filtered out before use.

Appendix 6. Preparation of Northern blot solutions

1. Solution D

250g guanidinium thiocyanate (Fluka) was dissolved in 293 ml pure water, to which 17.6 ml of 0.75 M sodium citrate pH 7.0 (previously prepared in a baked bottle and autoclaved) and 26.4 ml of 10% sarcosyl were then added. Before use, 0.36 ml Beta-mercaptoethanol (beta-ME) was added to 50 ml of this stock solution. Final solution was 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M beta-ME.

2. Phenol (water saturated)

100 g highly-purified Phenol (Gibco-BRL) stored at -20°C was melt in waterbath at 65°C and 250 ml of pure water was then added. After been shaken well, it was left overnight at 4°C to separate. Most of water was removed and the solution was stored at 4°C.

3. Sodium acetate (2M)

2 M sodium acetate (Sigma) was adjusted to pH 4.0 by using glacial acetic acid, and sterilised by autoclaving.

4. Chloroform/isoamyl alcohol

Chloroform/isoamyl alcohol (Sigma) was mixed in ratio 49:1 chloroform:isoamyl alcohol at RT.

5. 10X running buffer

8.36 g of MOPS, 0.744 g of EDTA and 0.82 g of sodium acetate were dissolved in 150 ml of pure water and the pH was adjusted to 7.0 with the sterile 5 M NaOH and made up to 200 ml with pure water. The final solution was 200 mM MOPS, 10 mM EDTA and 50 mM sodium acetate.

6. Sample buffer

The sample buffer was made up by mixing 200 μ l of 10X running buffer, 1 ml of deionized formamide and 356 μ l of 37% formaldehyde

7. Hybridization buffer (for 100 ml)

<u>Stock concentration</u>		<u>Final concentration</u>
1 M	sodium phosphate, pH 7.2	0.2 M
0.5 M	EDTA	1 mM
1g	BSA	1%
7g	SDS	7%
100%	Formamide	15%

After BSA was dissolved in the aqueous components, the SDS and formamide were added to the solution.

Appendix 7. Preparation of collagen coated multiwell plates

10 mg collagen (type I, Sigma) was dissolved in 0.1 M acetic acid at room temperature for 3 h. The solution was sterilized through a 0.2 μ m filter. The culture well surface was coated by collagen at 6 μ g/cm² and air-dried. Before introducing cells, the plates were rinsed with sterile water twice.

Appendix 8. Preparation of 40% Percoll Gradient

40% Percoll (Pharmacia Ltd, Milton Keynes, Bucks, UK) was obtained by mixing 9 ml Percoll with 1 ml of 10 x PRMI-1640 Medium (Flow Laboratories), which was further diluted by adding 12.5 ml of 1 x PRMI-1640 medium. The gradient was established by centrifugation at 13,000 g for 60 min in a fixed angle rotor.

Appendix 9. Preparation of plasmid cDNA of dopamine D₂ receptor

The dopamine D₂ receptor cDNA, either full length (2.5 kb) or short length (1.5 kb), was cloned into the E. coli site of plasmid pBluescript (Stratagene). The Miniprep Kit (Pharmacia LKB) was used to prepare and purify dopamine D₂ receptor cDNA from E. coli. The full length of dopamine D₂ receptor cDNA was used for constructing the probe for Northern blot or used for transfection and the short length of dopamine D₂ receptor cDNA coding from the third cytoplasmic loop to the 3' poly A tail was used for constructing ribo-probe for in situ hybridization.

A. Alkaline lysis miniprep

All of the solutions was included in Miniprep Kit (Pharmacia LKB, Biotechnology, Molecular Biology Division, S-75182 Uppsala, Sweden) except Terrific broth.

1. E.Coli cells, containing dopamine D₂ receptor inserts, either full length (2.5 kb) or short length (1.5 kb), were grown in 15 ml Terrific broth in a orbital shaker at 37°C (200 rpm/min) for 12 h;

2. The culture was aliquoted into 1.5 ml eppendorf tube, and centrifuged in a microcentrifuge at full speed for 1 min to pellet the cells;

3. After removing the medium by aspiration, 100 µl of **solutions I** (50 mM Tris-HCl (pH 8.0), 50 mM glucose, 10 mM EDTA, 0.15% kathon CG/ICP), 200 µl of **solution II** (0.8 M NaOH, 4% (v/v) Triton X-100) and

150 µl of **solution III** (3 M sodium acetate, pH 4.8), precooled on ice, were sequentially added to the tubes. After addition of each solution, the tubes were resuspended by vortexing and incubated on ice for 5 min;

4. The tubes were spun in a microcentrifuge at RT for 5 min at full speed;

5. The supernatant was carefully transferred to a clean microcentrifuge tube;

6. 450 µl of isopropanol was added. The solution was mixed by inverting and incubated at RT for 10 min;

7. The DNA was pelleted in a microcentrifuge at RT for 5 min at full speed and washed once with 250 µl of isopropanol;

8. The pellet was freeze-dried for 5 min and redissolved in 50 µl of column buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). DNA was then purified by spun columns included in Miniprep Kit (Pharmacia LKB). The preparation of spun column was performed according to the instruction of manufacture (Pharmacia LKB).

The purified DNA yield was estimated by comparing its fluorescence under ultraviolet light on 0.8% agarose gel with that of the three pBR322 samples.

Appendix 10. Restriction enzyme digestion

A. Digestion

To each eppendorf tube sitting on ice the following solutions (Northumbria, Biologicals Limited) were added:

31 µl plasmid cDNA

4 µl 10 X buffer H (for anti-sense) or buffer L (for sense)

5 µl Xba 1 (for anti-sense) or Kpn1(for sense)

The tubes were incubated in 37°C waterbath for 1 h. Then 1.5 µl of proteinase K was added to each tube and incubated in 37°C waterbath for 0.5 h.

B. Extraction of digested plasmid

By adding DEPC treated water to make the volume in each tube to 100 µl, DNA was then extracted with 100 µl phenol/choloroform twice and with 100 µl choloroform once, and then redissolved in 100 µl of DEPC treated water.

C. Precipitation

100 µl of DNA solution was added to a new tube which contained 200 µl of absolute alcohol and 20 µl of 10.5 M ammonia acetate. After precipitated at - 20°C for overnight, DNA solutions were pelleted in a microcentrifuge for 20 min at full speed. The pellet was then resuspended in 400 µl of 80% ethanol, centrifuged for 10 min at full speed. After freezing dry, the pellet was dissolved in 8 µl TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and left on ice for 20 min, then stored at -70°C until use.

The linearized plasmid DNA was checked on a 0.8% agarose gel and the yield of the plasmid DNA was estimated by comparison with pBR 322 standards.

Appendix 11. Ribo-probe labelling and hydrolysis

A. Labelling ribo-probe

T7 (antisense) and T3 (sense) RNA transcripts were synthesized and labelled with [³⁵S]UTP in vitro using the mCAP transcription kit (Molecular Biology Boehringer Mannheim).

The following reagents were added to the tube setting on the ice:

- 1 μ l RNase block
- 5 μ l 5x buffer
- 8 μ l DNA template (1 μ g)
- 1 μ l rATP
- 1 μ l rGTP
- 1 μ l rCTP
- 1 μ l rUTP (1/50 diluted)
- 1 μ l 0.75 M DTT
- 5 μ l [35 S]UTP
- 1 μ l T7 (for antisense) or T3 polymerase (for sense)

The final volume in each tube was made up to 25 μ l with DEPC treated water.

The tubes were incubated at 37°C for 30 min, and a further 1 μ l T7 or T3 polymerase was added and incubated at 37°C for 30 min. The solution was then treated with 1 μ l tRNA stock (20 μ g) and 1 μ l RNase free DNase at 37°C for 30 min. The volume was made up to 100 μ l with DEPC treated water, and then extracted twice with 100 μ l of buffered phenol/chloroform. The DNA was precipitated overnight with 10 μ l sodium acetate and 220 μ l ethanol at -70°C and pelleted in a microcentrifuge at full speed for 10 min at 4°C. The pellet was washed in 250 μ l cold 80% ethanol and centrifuged for 10 min. The supernatant was then decanted leaving a pellet of labelled RNA in the tube. After freezing dry, the pellet was reconstituted in 50 μ l DEPC treated water and left on ice for 20 min before assessing specific activity of the probe.

B. Probe Hydrolysis

The following solutions were incubated at 60°C for 70 min:

- 20 μ l labelled probe
- 380 μ l 50 mM DTT
- 380 μ l 1 M sodium bicarbonate
- 20 μ l 1 M DTT

The mixture was divided between two tubes. 114 μ l 10.5 M ammonium acetate and 400 μ l cold absolute alcohol were added to each tube. After precipitating at -20°C overnight, the DNA was pelleted by spinning at full speed for 20 min in a microcentrifuge and washed in 450 μ l 100% alcohol. The pellets were then freeze-dried briefly, redissolved in 25 μ l of 50 mM DTT, and left on ice for 20 min before accessing the specific activity of the probe.

Appendix 12. Deprotection of oligos

The human dopamine D2 receptor 3'primer was synthesized in 391 DNA Synthesizer (Applied Biosystems, PCR-MATE). On following day, the column C containing the synthesized oligonucleotides was removed and oligonucleotides was deprotected as follows:

1. The two ends of the column was tightly connected to sterile 10 ml syringes, one of which contained 2 ml of cold ammonia (stored at 4°C);
2. Ammonia was pushed through the column several times and then left in the column for 30 min before transferred to a 15 ml sterile tube;
3. The solution, capped and sealed with lab film, was incubated at 55°C waterbath overnight to deprotect the oligo;
4. 1/10 volume of 3 M sodium acetate, pH 7.0 and 2 volume of 100% ethanol were added to a 400 μ l aliquot of deprotected oligo. After precipitated at -70°C for 15 min, the oligo was pelleted by centrifugation at full speed for 10 min in a microcentrifuge;
5. The pellet was washed once with 500 μ l of 70% ethanol and freeze dried;
6. The freeze dried pellet was then resuspended in 200 μ l sterile water. The concentration of oligonuclei was determined by measuring O.D₂₆₀ of 10 μ l oligo solution in 1 ml distilled water.

- Alberts, B. Lewis, J., Raff, M., Roberts, K. and Walson, J.D. (1989).** Mechanisms of transduction by cell-surface receptor proteins. In: *Molecular Biology of the cell* (second edition), Garland Publishing, Inc., London, PP 693-708
- Al-Timimi, A. and Fox, H. (1986).** Immunohistochemical localisation of follicle-stimulating hormone, luteinizing hormone, growth hormone, adrenocorticotrophic hormone and prolactin in the human placenta. *Placenta* 7, 163-172
- Andersen, J.R. (1982).** Prolactin in amniotic fluid and internal serum during uncomplicated human pregnancy. *Dan. Med. Bull.* 29, 266-274
- Andersen, J.R., Borggaard, B., Ornvold, K. and Knoth, K. (1982).** The effect of hyposmotic and sodium chloride hyperosmotic environments on the secretion and synthesis of prolactin from human decidua in vitro. *Acta Endocr.* 100, 623-629
- Andersen, J.R., Borggaard, B., Schroeder, E., Olsen, E.B., Stimpel, H. and Nyholm, H.C. (1984).** The dependence of human decidual prolactin production and secretion on the osmotic environment in vitro. *Acta Endocr.* 106, 405-410
- Andersen, J.R., Borggaard, B., Olsen, E.B., Stimpel, H., Nyholm, H.C. and Schroeder, E. (1986).** Effect of ouabain and bumetanide on the basal and the osmolality-affected prolactin secretion from human decidual cells in vitro. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 23, 159-166
- Angerer, L.M., Stoler, M.H. and Angerer, R.C. (1987).** Chapter 3. In situ hybridization with RNA probes: An annotated recipe. In: Valentino, K.L., Eberwine, J.H. and Barchas, J.D. (eds.), *In Situ Hybridization*. Oxford University Press, Oxford, P29
- Bayard, F., Damilano, S., Robel, P. and Baulieu, E.E. (1978).** Cytoplasmic and nuclear estradiol and progesterone receptors in human endometrium. *J. Clin. Endocr. Metab.* 46, 635-648
- Bell, S.C. (1983).** Decidualization: regional differentiation and associated function. *Oxford Rev. Reprod. Biol.* 5, 220-271
- Bell, S.C. (1986).** Secretory endometrium and decidual proteins: studies and clinical significance of a maternally derived group of pregnancy-associated serum proteins. *Hum. Reprod.* 1, 129-143
- Bell, S.C. (1989).** Decidualization and insulin like growth factor (IGF) binding protein: implications for its role in stromal cell differentiation and the decidual cell in haemochorial placentation. *Hum. Reprod.* 4, 125-130

- Ben-David, M., Rodbard, D., Bates, R. W., Bridson, W. E. and Chrambach, A. (1973).** Human prolactin in plasma, amniotic fluid and pituitary: identity and characterization by criteria of electrophoresis and isoelectric focusing in polyacrylamide gel. *J. Clin. Endocr. Metab.* **36**, 951-964
- Ben-Jonathan, N. and Munsick, R.A. (1980).** Dopamine and prolactin in human pregnancy. *J. Clin. Endocr. Metab.* **51**, 1019-1025
- Ben-Jonathan, N. (1985).** Dopamine: a prolactin-inhibitor hormone. *Endocr. Rev.* **6**, 564-589
- Benedetto, M.T., Tabanelli, S. and Gurpide, E. (1990).** Estrone sulfate sulfatase activity is increased during in vitro decidualization of stromal cells from human endometrium. *J. Clin. Endocr. Metab.* **70**, 342-345
- Bergeron, C., Ferenczy, A. and Shyamala, G. (1988a).** Distribution of estrogen receptor in various cell types of normal, hyperplastic, and neoplastic human endometrial tissues. *Lab. Invest.* **58**, 338-345
- Bergeron, C., Ferenczy, A., Toft, D.O., Schneider, W. and Shyamala, G. (1988b).** Immunocytochemical study of progesterone receptors in the human endometrium during the menstrual cycle. *Lab. Invest.* **59**, 862-869
- Bigazzi, M., Ronga, R., Lancranjan, I., Ferraro, S., Branconi, F., Buzzoni, P., Martorana, G., Scarselli, G. F. and Pozo, E. D. (1979a).** A pregnancy in an acromegalic woman during bromocriptine treatment: effects on growth hormone and prolactin in the maternal, fetal, and amniotic compartments. *J. Clin. Endocr. Metab.* **48**, 9-12
- Bigazzi, M., Pollicino, G. and Nardi, E. (1979b).** Is human decidua a specialised endocrine organ? *J. Clin. Endocr. Metab.* **49**, 847-850.
- Birge, C.A., Jacobs, L.S., Hammer, C.T. and Daughaday, W.H. (1970).** Catechalamine inhibition of prolactin secretion by isolated rat adenohypophyses. *Endocrinology* **86**, 120-126
- Bischof, P., Sizonenko, M. T. and Herrmann, W. L. (1986).** Trophoblastic and decidual response to RU486: effects on human chorionic gonadotropin, human placental lactogen, prolactin and pregnancy-associated plasma-protein-A production in vitro. *Hum. Reprod.* **1**, 3-6
- Boenisch, T. (1990).** Background. In: Naish, S.J. (ed), Immunocytochemical staining methods, pp 21-23

- Braverman, M.B., Bagni, A., DeZeigler, D., Den, T. and Gurside, E.** (1984). Isolation of prolactin-producing cells from first and second trimester decidua. *J. Clin. Endocr. Metab.* **58**, 521-525
- Braverman, M.B., and Gurside, E.** (1986). In vitro effects of human prolactin and oxytocin on sulfatase activity in isolated human decidual cells. *J. Clin. Endocr. Metab.* **63**, 725-729
- Brenner, R. M., West, N.B. and McClellan, M.C.** (1990). Estrogen and progesterin receptors in the reproductive tract of male and female primates. *Biol. Reprod.* **42**, 11-19
- Bryant-Greenwood, G.D., Rees, M.C.P. and Turnbull, A.C.** (1987). Immunohistochemical localisation of relaxin, prolactin and prostaglandin synthase in human amnion, chorion and decidua. *J. Endocr.* **114**, 491-496
- Butzow, R., Alfthan, H., Julkunen, M., Rutanen, E. M., Bohn, H. and Seppala, M** (1986). Human endometrium and menstrual fluid contain placental protein 5 (PP5). *Hum. Reprod.* **1**, 287-289
- Caron, M.G., Beaulieu, M., Raymond, V., Gagne, B., Drouin, J., Lefkowitz, R.J. and Labrie, F.** (1978). Dopaminergic receptors in the anterior pituitary gland. Correlation of [3 H] dihydroergocryptine binding with the dopaminergic control of prolactin release. *J. Biol. Chem.* **253**, 2244-2253
- Casslen, B.G., Siler-Khodr, T.M. and Harper, M.J.K.** (1990). Progesterone regulation of prolactin release from human endometrial stromal cells in culture: Potential bioassay for progestational activity. *Acta Endocr.* **123**, 137-144
- Chen, G., Huang, J. R., Mazella, J. and Tseng, L.** (1989). Long-term effects of progestin and RU486 on prolactin production and synthesis in human endometrial stromal cells. *Hum. Reprod.* **4**, 355-358.
- Chomczynski, P. and Sacchi, N.** (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
- Clements, C., Whitfeld, P., Cooke, N.E., Healy, D., Matheson, B., Shine, J. and Funder, J.** (1983). Expression of the prolactin gene in human decidua chorion. *Endocrinology* **112**, 1133-1134
- Cook, N.E., Coit, D., Shine, J., Baxter, J.D. and Martial, J.A.** (1981). Human prolactin cDNA structural analysis and evolutionary comparisons. *J. Biol. Chem.* **256**, 4007-4016
- Dallenbach-Hellweg, G.** (1988). The endometrium in natural and artificial luteal phases. *Hum. Reprod.* **3**, 165-168

- Daly, D. C., Maslar, I. A., Rosenberg, S.M., Tohan, N. and Riddick, D. H. (1981). Prolactin production by luteal phase endometrium. *Am. J. Obstet. Gynecol.* 140, 587-591
- Daly, D. C., Maslar, I. A. and Riddick, D. H. (1983a). Prolactin production during in vitro decidualization of proliferative endometrium. *Am. J. Obstet. Gynecol.* 145, 672-678.
- Daly, D.C., Maslar, I.A. and Riddick, D.H. (1983b). Term decidua response to estradiol and progesterone. *Am. J. Obstet. Gynecol.* 145, 679-683
- Davis, J.R.E. (1990). Tissue-specific regulation of prolactin gene expression. *J. Endocr.* 125, 171-173
- DeZeigler, D. and Gurside, E. (1982). Production of prolactin by cultures of cells from human decidua. *J. Clin. Endocr. Metab.* 55, 511-515
- DeFeo, V.J. (1967). Decidualisation. In: Wynn, R.M. (ed), *Cellular Biology of the Uterus*, Division of Meredith Publishing Company, New York. PP 191-276
- Diani, F. and Perdelli, F. (1981). Amniotic fluid prolactin and fetal lung maturity. *Clin. Exp. Obstet. Gynecol.* VIII, 50-53
- DiMattia, G. E., Gellersen, B., Duckworth, M. L. and Friesen, H. G. (1990). Human prolactin gene expression. The use of an alternative noncoding exon in decidua and the IM-9-P3 lymphoblast cell line. *J. Biol. Chem.* 265, 16412-16421
- Eckert, R.I. and Katzenellenbogen, B.S. (1981). Human endometrial cells in primary culture: modulation of the progesterone receptor level by natural and synthetic oestrogen in vitro. *J. Clin. Endocrinol. Metab.* 52, 699-708
- Elazar, Z., Siegel, G. and Fuchs, S. (1989). Association of two pertussis toxin-sensitive G-proteins with the D2-dopamine receptor from bovine striatum. *EMBO J.* 8, 2353-2357
- Elsholtz, H.P., Mangalam, H.J. and Potter, E. (1986). Two different cis-active elements transfer the transcriptional effects of both EGF and phorbol esters. *Science* 234, 1552-1557
- Enders, A.C., Welsh, A.O. and Schlafke, S. (1985). Implantation in the rhesus monkey: Endometrial responses. *Am. J. Anat.* 173, 147-169
- Fang, V.S. and Kim, M.H. (1975). Study on maternal, fetal and amniotic human prolactin at term. *J. Clin. Endocr. Metab.* 41, 1030-1034

- Farmilo, A.J. and Stead, R.H. (1990).** Fixation in immunocytochemistry. In: Naish, S.J. (ed), Immunochemical staining methods, pp 24-29
- Feil, P.D. and Bardin, C.W. (1975).** Cytoplasmic and nuclear progesterone receptors in the guinea pig uterus. *Endocrinology* **97**, 1398-1407
- Feinberg, A.P. and Vogelstein, B. (1983).** A technique for labelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13
- Findlay, J.K., Salamonsen, L.A. and Cherny R.A. (1990).** Endometrial functions: Studies using isolated cells in vitro. *Oxford Rev. Reprod. Biol.* **12**, 181-223
- Finn, C. A. (1977).** The implantation reaction, In: Wynn R. M. (ed), *Biology of the Uterus*, Plenum Press, London, P 292.
- Fox, C.H., Johnson F.B., Whiting J. and Roller P.P. (1985).** Formaldehyde fixation. *J. Histochem. Cytochem.* **33**, 845-853
- Frame, L.T., Rogol, A.D., Riddick, D.H. and Baczynski, E. (1979a).** Gel chromatographic properties of human prolactin released from decidua tissue in vitro. *Fertil. Steril.* **31**, 647-650
- Frame, L. T., Wiley, L. and Rogol, A. D. (1979b).** Indirect immunofluorescent localisation of prolactin to the cytoplasm of decidua and trophoblast cells in human placental membranes at term. *J. Clin. Endocr. Metab.* **49**, 435-437
- Ganong, W.F. (1984).** Physiology of the female reproductive system. In: Benson, R.C. (ed), *Current Obstetric and Gynecologic diagnosis and treatment* (5th edition), Lange Medical Publications, California, P 81
- Garcia, E., Bouchard, P., DeBrux, J., Berdah, J., Frydman, R., Schaison, G., Milgrom, E. and Perrot-Applanat, M. (1988).** Use of immunocytochemistry of progesterone and estrogen receptors for endometrial Dating. *J. Clin. Endocr. Metab.* **67**, 80-87
- Gellersen, B., DiMattia, G. E., Friesen, H. G. and Bohnet, H. G. (1989a).** Prolactin (PRL) mRNA from human decidua differs from pituitary PRL mRNA but resembles the IM-9-P3 lymphoblast PRL transcript. *Mol. Cell. Endocr.* **64**, 127-130
- Gellersen, B., DiMattia, G.E., Friesen, H.G. and Bohnet, H.G. (1989b).** Phorbol ester stimulates prolactin release but reduces prolactin mRNA in the human B-lymphoblastoid cell line IM-9-P3. *Mol. Cell. Endocr.* **66**, 153-161

- Ghosh, D. and Sengupta, J. (1988).** Patterns of estrogen and progesterone receptors in rhesus monkey endometrium during secretory phase of normal menstrual cycle and preimplantation stages of gestation. *J. Steroid. Biochem.* **31**, 223-229
- Glasser, S. R. (1986).** Current concept of implantation and decidualization In: Huszar, G. (ed), The physiology and biochemistry of the uterus in pregnancy and labour. CRC Press, Inc., Florida. PP128-161
- Golander, A., Hurley, T., Barrett, J., Hizi, A. and Handwerger, S. (1978).** Prolactin synthesis by human chorion-decidual tissue: a possible source of prolactin in the amniotic fluid. *Science* **202**, 311-312
- Golander, A., Barrett, J., Hurley, T., Barry, S. and Handwerger, S. (1979).** Failure of bromocriptine, dopamine, and thyrotropin-releasing hormone to affect prolactin secretion by human decidual tissue in vitro. *J. Clin. Endocr. Metab.* **49**, 787-789
- Golander, A., Zakuth, V., Shechter, Y. and Spirer, Z. (1984).** Suppression of lymphocyte reactivity in vitro by a soluble factor secreted by explants of human decidua. *Eur. J. Immunol.* **11**, 849-851
- Golander, A., Richards, R., Thraikill, K., Capel, D., Rogers, D. and Handwerger, S. (1988).** Decidual prolactin (PRL)-releasing factor stimulates the synthesis of PRL from human decidual cells. *Endocrinology* **123**, 335-339
- Gorodeski, I.G., Bahary, C.M., Beery, R., Lunenfeld, B. and Geier, A. (1987).** Characterization of the nuclear progesterone receptor in normal human endometrium after progesterone injection. *Gynecol. Obstet. Invest.* **23**, 48-53
- Gorodeski, I.G., Bahary, C.M., Lunenfeld, B., Beery R. and Geier, A. (1988).** Distribution of total progesterone receptor levels in various segments and tissues of the normal human uterus: the effect of short-term estrogen administration. *J. Endocr. Invest.* **11**, 465-469
- Gorski, J., Toft, D., Shyamala, G., Smith, D. and Notides, A. (1968).** Hormonal receptors: studies on the interaction of estrogen with the uterus. *Recent Prog. Horm. Res.* **24**, 45-79
- Gorski, J. and Gannon, F. (1976).** Current models of steroid hormone action: a critique. *Ann. Rev. Physiol.* **38**, 425-450
- Gorski, J., Welshons, W. V., Sakai, D., Hansen, J., Walent, J., Kassis, J., Shull, J., Stack, G. and Campen, C. (1986).** Evolution of a model of estrogen action. *Recent Prog. Horm. Res.* **42**, 297-329

- Grosso, D.S., MacDonald, C.P., Thomasson, J.E. and Christian, C.D. (1980). Relationship of newborn serum prolactin levels to the respiratory distress syndrome and maternal hypertension. *Am. J. Obstet. Gynecol.* **137**, 569-575
- Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Appianta, M. and Milgrom, E. (1989). Mechanism of nuclear localisation of the progesterone receptor: evidence for interaction between monomers. *Cell* **57**, 1147-1154
- Haluska, G.J., West, N.B., Novy, M.J. and Brenner, R.M. (1990). Uterine estrogen receptors are increased by RU486 in late pregnant rhesus macaques but not after spontaneous labour. *J. Clin. Endocr. Metab.* **70**, 181-186
- Hamosh, M. and Hamosh, P. (1977). The effect of prolactin on the lecithin content of fetal rabbit lung. *J. Clin. Invest.* **59**, 1002-1005
- Handwerger, S., Barry, S., Barrett, J., Markoff, E., Zeitler, P., Cwikel, B. and Siegel, M. (1981). Inhibition of the synthesis and secretion of decidual prolactin by arachidonic acid. *Endocrinology* **109**, 2016-2021
- Handwerger, S., Barry, S., Markoff, E., Barrett, J. and Conn, P.M. (1983). Stimulation of the synthesis and release of decidual prolactin by a placental polypeptide. *Endocrinology* **112**, 1370-1374
- Handwerger, S., Wilson, S. and Conn, P.M. (1984). Different subcellular storage sites for decidual- and pituitary-derived prolactin: possible explanation for differences in regulation. *Mol. Cell. Endocr.* **37**, 83-87
- Handwerger, S. and Capel, W. D. (1985). Differences in the cell surface antigens of prolactin-producing cells of human decidual and pituitary tissues. *J. Clin. Endocr. Metab.* **61**, 830-833
- Handwerger, S., Capel, W. D., Korner, G. and Richards, R. (1987a). Purification of decidual prolactin-releasing factor, a placental protein that stimulates prolactin release from human decidual tissue. *Biochem. Biophys. Res. Comm.* **147**, 452-459
- Handwerger, S., Harman, I., Costello, A. and Markoff, E. (1987b). Cyclic AMP inhibits the synthesis and release of prolactin from human decidual cells. *Mol. Cell. Endocr.* **50**, 99-106
- Harman, I., Costello, A., Ganong, B., Bell, R. M. and Handwerger, S. (1986). Activation of protein kinase C inhibits synthesis and release of decidual prolactin. *Am. J. Physiol.* **251** (Endocr Metab 14), E172-E177

- Hartman, B.K. (1973).** Immunofluorescence of dopamine β -hydroxylase. Application of improved methodology to the localization of the peripheral and central noradrenergic nervous system. *J. Histochem. Cytochem.* **21**, 312-332
- Healy, D. L., Muller, H. K. and Burger, H. G. (1977).** Immunofluorescence shows localization of prolactin to human amnion. *Nature* **265**, 642-643
- Healy, D. L., Kimpton, W. G., Muller, H. K. and Burger, H. G. (1979).** The synthesis of immunoreactive prolactin by decidua-chorion. *Br. J. Obstet. Gynaecol.* **86**, 307-313
- Healy, D. L., Herington, A. C. and O'Herlily, C. (1982).** Chronic idiopathic polyhydramnios: evidence for a defect in chorion laeve receptor for lactogenic hormones. *J. Clin. Endocr. Metab.* **56**, 520-523
- Healy, D. L. (1984).** The clinical significance of endometrial prolactin. *Aust. NZ J. Obstet. Gynec.* **24**, 111-116
- Healy, D. L., Herington, A. C. and O'Herlily, C. (1985).** Chronic polyhydramnios is a syndrome with a lactogen receptor defect in the chorion laeva. *Br. J. Obstet. Gynaecol.* **92**, 461-467
- Herington, A. C., Graham, J. and Healy, D. L. (1980).** The presence of lactogen receptors in human chorion laeve. *J. Clin. Endocr. Metab.* **51**, 1466-1468
- Hiraoka, Y., Tatsumi, K., Shiozawa, M., Aiso, S., Fukasawa, T., Yasuda, K. and Miyai, K. (1991).** A placenta-specific 5'non-coding exon of human prolactin. *Mol. Cell. Endocr.* **75**, 71-80.
- Hochner-Celnikier, D., Ron, M., Eldor, A., Segal, S., Palti, Z., Fuks, Z. and Vlodavsky, I. (1984).** Growth characteristics of human first trimester decidual cells cultured in serum-free medium: production of prolactin, prostaglandins and fibronectin. *Biol. Reprod.* **31**, 827-836
- Holt, W. F. and Perks, A. M. (1975).** The effect of prolactin on water movement through the isolated amniotic membrane of the guinea pig. *Gen. Comp. Endocr.* **26**, 153-164
- Horrobin, D.R. (1973).** An inhibitory effect of prolactin on the response of rat myometrium to oxytocin. *Experientia* **29**, 109-110
- Huang, J.R., Tseng, L., Bischof, P. and Janne, O.A. (1987).** Regulation of prolactin production by progestin, estrogen, and relaxin in human endometrial stromal cells. *Endocrinology* **121**, 2011-2017

- Illingworth, D.V., Wood, G.P., Flickinger, G.L. and Mikhail, G. (1971).** Progesterone receptor of the human myometrium. *J. Clin. Endocr. Metab.* **40**, 1001-1008
- Innis, M.A. and Gelfand, D.H. (1990).** Optimization of PCRs, In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds), *PCR Protocols: A Guide to Methods and Applications*, academic press, Inc., London, PP 3-12
- Ingram, C.D., Bicknell, R.J. and Mason, W.T. (1986).** Intracellular recordings from bovine anterior pituitary cells: Modulation of spontaneous activity by regulation of prolactin secretion. *Endocrinology* **119**, 2508-2518
- Irwin, J.C., Quigley, M.M., Kirk, D., Gwatkin, R.B.L. and King, R.J. (1989).** Hormonal regulation of human endometrial stromal cells in culture: an in vitro model for decidualization. *Fertil. Steril.* **52**, 761-768
- sJackson, A.E. and Bancraft, C. (1988).** Proximal upstream flanking sequences direct calcium regulation of rat prolactin gene. *Mol. Endocr.* **2**, 1139-1144
- Jayatilak, P.G. and Gibori, G. (1986).** Ontogeny of prolactin receptors in rat decidua tissue: binding by a locally produced prolactin-like hormone. *J. Endocr.* **110**, 115-121
- Jensen, E.V. and Jacobson, H.I. (1962).** Basic guides to the mechanism of oestrogen action. *Recent Prog. Horm. Res.* **18**, 387-414
- Jensen, E.V. and DeSombre, E.R. (1972).** Mechanism of action of the female sex steroids. *Ann. Rev. Biochem.* **41**, 203-229
- Jensen, E.V. and DeSombre, E.R. (1973).** Estrogen receptor interaction. *Science* **182**, 126-128
- Johnson, J. W. C., Tyson, J. E., Mitzner, W., Beck, J. C., Andreassen, B., London, W. T. and Villar, J. (1985).** Amniotic fluid prolactin and fetal lung maturation. *Am. J. Obstet. Gynecol.* **153**, 372-380
- Joshi, S.G., Ebert, K. M. and Smith, R.A. (1980).** Properties of the progesterone-dependent protein of the human endometrium. *J. Reprod. Fert.* **59**, 287-296
- Josimovich, J.B., Weiss, G. and Hutchinson, D.C. (1974).** Sources and disposition of pituitary prolactin in maternal circulation, amniotic fluid, fetus and placenta in the pregnant rhesus monkey. *Endocrinology* **94**, 1364-1371

- Josimovich, J.B., Merisko, K. and Boccella, L. (1977).** Amniotic prolactin control over amniotic and fetal extracellular fluid water and electrolytes in the rhesus monkey. *Endocrinology* **100**, 564-570
- Julkunen, M. (1986).** Human decidua synthesis placental 14 (PP14) in vitro. *Acta Endocrinology* **112**, 271-277
- Karmali, R.A., Lander, I. and Horrobin, D.F. (1974).** Prolactin and the immune response. *Lancet* **2**, 106-107
- Kasai, K., Shik, S.S. and Yoshida, Y. (1982).** Production and localisation of human prolactin in placenta and decidua in early and at term normal pregnancy. *Biol. Res. Pregnancy* **3**, 25-29
- Kauma, S. and Shapiro, S.S. (1986).** Immunoperoxidase localization of prolactin in endometrium during normal menstrual, luteal phase defect, and corrected luteal phase defect cycles. *Fertil. Steril.* **46**, 37-41
- Kawasaki, E.S. (1990).** Amplification of RNA. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds.), *PCR Protocols: A Guide to Methods and Applications*, academic Press, Inc., London, P 21
- Kearns, M. and Lala, P.K. (1983).** Life history of decidual cells: a review. *Am. J. Reprod. Immunol. Microbiol.* **3**, 78-82
- Kebabian, J.W. and Calne, D.B. (1979).** Multiple receptors for dopamine. *Nature* **277**, 90-93
- Khan-Dawood, F.S. and Dawood, M.Y. (1984).** Estrogen and progesterone receptor and hormone levels in human myometrium and placenta in term pregnancy. *Am. J. Obstet. Gynecol.* **150**, 501-505
- King, R.J.B., Gordon, J., and Inman, D.R. (1965).** The intracellular localization of oestrogen in rat tissues. *J. Endocr.* **32**, 9-15
- King, W.J. and Greene, G.L. (1984).** Monoclonal antibodies localise oestrogen receptor in the nuclei of target cells. *Nature* **307**, 745-747
- Kishimoto, Y., Tominaga, T., Aso, T., Kinoshita, M. and Mori, T. (1987).** Human trophoblast and endometrial interactions in vitro. *Acta Obst. Gynaec. Jpn.* **39**, 463-470
- Kletzky, O.A., Rossman, F., Bertolli, S.I., Platt, L.D. and Mishell, D.R. (1985).** Dynamics of human chorionic gonadotropin, prolactin, and growth hormone in serum and amniotic fluid throughout normal human pregnancy. *Am. J. Obstet. Gynecol.* **151**, 878-884

- Koistinen, R., Kalkkinen, N., Huhtala, M.L., Seppala, M., Bohn, H. and Rutanen, E.M. (1986). Placental protein 12 is a decidual protein that binds somatomedin and has an identical N-terminal amino acid sequence with somatomedin-binding protein from human amniotic fluid. *Endocrinology* 118, 1375-1378
- Kreitmunn-Gimbal, B., Bugat, R. and Bayard, F. (1979). Estrogen and progestin regulation of the progesterone receptor concentration in human endometrium. *J. Clin. Endocr. Metab.* 49, 926-929
- Kreitmunn-Gimbal, B., Bayard, F., Nixon, W.E. and Hodgen, G.O. (1980). Patterns of estrogen and progesterone receptors in monkey endometrium during the normal menstrual cycle. *Steroids* 35, 471-478
- Lawn, A.M., Wilson, E. W. and Finn, C.A. (1971). The ultrastructure of human decidual and predecidual cells. *J. Reprod. Fertil.* 26, 85-90
- Lawrence, J.B. and Singer, R.H. (1985). Quantitative analysis of in situ hybridization methods for the detection of actin gene expression. *Nucleic Acids Res.* 13, 1777-1798
- Leontic, E.A. and Tyson, J.E. (1977). Prolactin and fetal osmoregulation: water transport across isolated human amnion. *Am. J. Physiol.* 232, R124-R127
- Lessey, B.A., Killam, A.P., Metzger, D.A., Haney, A.F., Greene, G.L. and McCarty, K.S. (1988). Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J. Clin. Endocr. Metab.* 67, 334-340
- Levy, C., Robet, P., Gautray, J.P., DeBrux, J., Verma, U., Descomps, B. and Baulieu, E.E. (1980). Estradiol and progesterone receptors in human endometrium: normal and abnormal menstrual cycles and early pregnancy. *Am. J. Obstet. Gynecol.* 136, 646-651
- Lubbert, H., Pollow, A., Rommler, A. and Hammerstein, J. (1982). Estradiol and progesterone receptor concentrations and 17 β -hydroxysteroid-dehydrogenase activity in estrogen-progestin stimulated endometrium of women with gonadal dysgenesis. *J. Steroid. Biochem.* 17, 143-148
- MacLeod, R.M. and Lehmeyer, J.E. (1974). Studies on the mechanism of the dopamine-mediated inhibition of prolactin secretion. *Endocrinology* 94, 1077-1085
- Markoff, E., Barry, S. and Handwerger, S. (1982). Influence of osmolality and ionic environment on the secretion of prolactin by human decidua in vitro. *J. Endocr.* 92, 103-110

- Markoff, E., Zeitler, P., Peleg, S. and Handwerger, S. (1983a)** Characterization of the synthesis and release of prolactin by an enriched fraction of human decidual cells. *J. Clin. Endocr. Metab.* **56**, 962-968
- Markoff, E., Howell, S. and Handwerger, S. (1983b).** Inhibition of decidual prolactin release by a decidual peptide. *J. Clin. Endocr. Metab.* **57**, 1282-1286
- Maslar, I. A. and Riddick, D.H. (1979).** Prolactin production by human endometrium during the normal menstrual cycle. *Am. J. Obstet. Gynecol.* **135**, 751-754.
- Maslar, I. A., Kaplan, B. M., Luciano, A. A. and Riddick, D. H. (1980).** Prolactin production by the endometrium of early pregnancy. *J. Clin. Endocr. Metab.* **51**, 78-83
- Maslar, I.A., Powers-craddock, P. and Ansbacher, R. (1986).** Decidual prolactin production by organ cultures of human endometrium: Effects of continuous and intermittent progesterone treatment. *Biol. Reprod.* **34**, 741-750
- Maslar, I.A. and Ansbacher, R. (1986).** Effects of progesterone on decidual prolactin production by organ cultures of human endometrium. *Endocrinology* **118**, 2102-2108
- Maslar, I.A., Lazur, J.J., Norman, R.I., and Spies, H.G. (1988).** Amniotic fluid and decidual prolactin during pregnancy in rhesus macaques. *Biol. Reprod.* **38**, 1067-1076
- Maslar, I.A. and Ansbacher, R. (1988).** Effect of short-duration progesterone treatment on decidual prolactin production by cultures of proliferative human endometrium. *Fertil. Steril.* **50**, 250-254
- Maurer, R.A. (1982).** Adenosine 3',5'-monophosphate derivatives increase prolactin synthesis and prolactin messenger ribonucleic acid levels in ergocryptine-treated pituitary cells. *Endocrinology* **110**, 1957-1963
- McCoshen, J.A., Tagger, O.Y., Wodzicki, A. and Tyson, J.E. (1982a).** Choriondecidual adhesion promotes decidual prolactin transport by human fetal membrane. *Am. J. Physiol.* **243**, R552-R557
- McCoshen, J.A., Tomita, K., Fernandez, C. and Tyson, J.E. (1982b).** Specific cells of human amnion selectively localise prolactin. *J. Clin. Endocr. Metab.* **55**, 166-169
- McCoshen, J.A. and Barc, J. (1985).** Prolactin bioactivity following decidual synthesis and transport by amniochorion. *Am. J. Obstet. Gynecol.* **153**, 217-223

- McNeilly, A.S., Gilmore D., Jeffery D., Dobbie G. and Chard T. (1977).** The origin of prolactin in amniotic fluid. In: Crosignani P. G. and Robyn C. (eds), Prolactin and human reproduction. Proceeding of the seronosymposia, Volume II, London, Academic Press, PP 21-26
- McNeilly, A.S. and Fraser, H.M. (1987).** Effect of GnRH agonist-induced suppression of LH and FSH on follicle growth and corpus luteum function in the ewe. *J. Endocr.* 115, 273-282
- Mcwey, L.A., Singhas, C.A. and Royol, A.D. (1982).** Prolactin binding sites on human chorion-decidua tissue. *Am. J. Obstet. Gynecol.* 144, 283-288
- Meador-Woodruff, J.H., Mansour, A., Bunzow, J.R., VanTol, H.H.M., Watson, S.J. and Civelli, O. (1989).** Distribution of D2 dopamine receptor mRNA in rat brain. *Proc. Natl. Acad. Sci. USA* 86, 7625-7628
- Meinkoth, J. and Wahl, G. (1984).** Review: Hybridization of nucleic acids immobilized on solid supports. *Anal. Biochem.* 138, 267-284
- Mendelson, C.R., Johnston, J.M., MacDonald, P.C. and snyder, J.M. (1981).** Multihormonal regulation of surfactant synthesis by human fetal lung in vitro. *J. Clin. Endocr. Metab.* 53, 307-317
- Meuris, S., Soumenkoff, G., Malengreall, A., and Robyn, C. (1980).** Immunoenzymatic localization of prolactin-like immunoreactivity in decidual cells of the endometrium from pregnant and non-pregnant women. *J. Histochem. Cytochem.* 28, 1347-1350
- Morimoto, Y. (1988).** Ultrastructural and endocrinological study of the human endometrium and decidua using the organ culture method. *Acta Obst. Gynaec. Jpn.* 40, 201-208
- Mueller, G.C., Vonderhaar, B., Kim, U.H. and Mahieu, M. (1972).** Estrogen action: an inroad to cell biology. *Recent Prog. Horm. Res.* 28, 1-49
- Murdoch, G.H., Waterman, M., Evans, R.M. and Rosenfeld, M.G. (1985).** Molecular mechanism of phorbol ester, thyrotropin-releasing hormone, and growth factor stimulation of prolactin gene transcription. *J. Biol. Chem.* 260, 11852-11858
- Nuwayhid, B. (1979).** Hemodynamic changes during pregnancy in the rabbit. *Am. J. Obstet. Gynecol.* 135, 590-596
- Ohara, K., Haga, K., Berstein, G., Haga, T., Ichiyama, A. and Ohara, K. (1988).** The interaction between D-2 dopamine receptors and GTP binding proteins. *Mol. Pharmacol.* 33, 290-296

- Okulicz, W.C., Savasta, A.M., Hoberg, L.M. and Longcope, C. (1989).** Immunofluorescent analysis of estrogen induction of progesterone receptor in the rhesus uterus. *Endocrinology* 125, 930-934
- O'Malley, B.W., Toft, D.O. and Sherman, M.R. (1971).** Progesterone-binding components of chick oviduct II nuclear components. *J. Biol. Chem.* 246, 1117-1122
- Oshorne, R. and Tashjian, A.H. (1981).** Tumor-promoting phorbol esters affect production of prolactin and growth hormone by rat pituitary cells. *Endocrinology* 108, 1164-1170
- Padayachi, T., Pegoraro, R.J., Hofmeyr, J., Joubert, S.M. and Norman, R.J. (1987).** Decreased concentrations and affinities of oestrogen and progesterone receptors of intrauterine tissue in human pregnancy. *J. Steroid. Biochem.* 26, 473-479
- Pavlik, E. and Katzenellenbogen, B.S. (1978).** Human endometrial cells in primary tissue culture: estrogen interaction and modulation of cell proliferation. *J. Clin. Endocr. Metab.* 47, 333-344
- Perrot-Applanat, M., Logeat, F., Groyer-picard, M.T. and Milyrom, E. (1985).** Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. *Endocrinology* 116, 1473-1484
- Perrot-Applanat, M., Groyer-picard, M.T., Logeat, F. and Milgrom, E. (1986).** Ultrastructural localisation of the progesterone receptor by an immunogold method: effect of hormone administration. *J. Cell. Biol.* 102, 1191-1199
- Perrot-Applanat, M., Groyer-Picard, M.T., Garcia, E., Lorenzo, F. and Milgrom, E. (1988).** Immunocytochemical demonstration of estrogen and progesterone receptors in muscle cells of uterine arteries in rabbits and humans. *Endocrinology* 123, 1511-1519
- Peter, S.T., Taylor, P.L. and Eidne, K.A. (1991).** Cellular localization of seven transmembrane domain receptor mRNA's by in situ hybridization. *Series H: Cell Biology* 52, 115-129
- Pihoker, C., Feeney, R.J., Su, Ju-Lan and Handwerger, S. (1991).** Lipocortin-I inhibits the synthesis and release of prolactin from human decidual cells: evidence for autocrine/paracrine regulation by lipocortin-I. *Endocrinology* 128, 1123-1128
- Pileri, S., Turi, A. and Rivano, M. T. (1982).** Immunohistochemical identification of prolactin in human placenta and membranes at term. *Clin. Exp. Obstet. Gynecol.* 9, 74-77
- Press, M.F. and Greene, G.L. (1984).** Methods in Laboratory Investigation. An immunocytochemical method for demonstrating

estrogen receptor in human uterus using monoclonal antibodies to human estrophilin. *Lab. Invest.* 50, 480-486

- Press, M.F. and Greene, G.L. (1988).** Localization of progesterone receptor with monoclonal antibodies to the human progestin receptor. *Endocrinology* 122, 1165-1175
- Press, M.F., Udove, J.A. and Greene, G.L. (1988).** Progesterone receptor distribution in the human endometrium. *Am. J. Pathol.* 131, 112-124
- Pritchard, J.A., MacKonald, P.C. and Gant, N.F. (1985).** The placenta and fetal membrane. In: Williams Obstetrics (Seventeenth edition), Appleton-Century-Crofts, a publishing division of Prentice-Hall, Inc., PP 97-117
- Raabe, M.A. and McCoshen, J.A. (1986).** Epithelial regulation of prolactin effect on amniotic permeability. *Am. J. Obstet. Gynecol.* 154, 130-134
- Randolph, J.F., Peegel, H., Ansbacher, R. and Menon, K.M.J. (1990).** In vitro induction of prolactin production and aromatase activity by gonadal steroids exclusively in the stroma of separated proliferative human endometrium. *Am. J. Obstet. Gynecol.* 162, 1109-1114
- Ren, S.G. and Braunstein G.D. (1990).** Progesterone and human chorionic gonadotropin do not stimulate placental proteins 12 and 14 or prolactin production by human decidual tissue in vitro. *J. Clin. Endocr. Metab.* 70, 983-989
- Resnik, R. (1986).** Regulation of uterine blood flow. In: Huszar, G. (ed), The physiology and biochemistry of the uterus in pregnancy and labour. CRC Press, Boca Raton, FL, P 25
- Richards, S.R., Kim, M.H. and Malarkey, W.B. (1982).** Evidence that chorion-decidual prolactin release is calcium dependent. *J. Clin. Endocr. Metab.* 54, 820-823
- Riddick, D. H. and Kusmik, W. F. (1977).** Decidua: a possible source of amniotic fluid prolactin. *Am. J. Obstet. Gynecol.* 127, 187-190.
- Riddick, D.H., Luciano, A.A., Kusmik, W.F. and Maslar, I.A. (1978).** De Novo synthesis of prolactin by human decidua. *Life Sciences* 23, 1913-1922
- Riddick, D.H. and Maslar, I.A. (1981).** The transport of prolactin by human fetal membranes. *J. Clin. Endocr. Metab.* 52, 220-224
- Rosenberg, S.M., Maslar, I.A. and Riddick, D.H. (1980).** Decidual production of prolactin in late gestation: further evidence for a

- decidual source of amniotic fluid prolactin. *Am. J. Obstet. Gynecol.* 138, 681-685
- Rosenberg, S.M. and Bhatnagar, A.S. (1984).** Sex steroid and human chorionic gonadotropin modulation of in vitro prolactin production by human term decidua. *Am. J. Obstet. Gynecol.* 148, 461-465
- Rosenberg, S.M. (1984).** Amniotic fluid prolactin levels in tubal gestation: support for the unique role of decidua. *Am. J. Obstet. Gynecol.* 150, 102-103
- Sakamoto, H., Maclusky, N. and Naftolin, F. (1986).** Steroid receptors in the pregnant uterus. In: Huszar, G. (ed), *The physiology and biochemistry of the uterus in pregnancy and labour.* CRC Press, Inc., Florida. PP 35-51
- Savouret, J.F., Misrahi, M. and Milgrom, E. (1988).** Molecular action of progesterone. *Oxford Rev. Reprod. Biol.* 10, 293-347
- Schenker, J. G., Ben-David, M. and Polishuk, W. Z. (1975).** Prolactin in normal pregnancy: relationship of maternal, fetal, and amniotic fluid levels. *Am. J. Obstet. Gynecol.* 123, 834-838
- Senogles, S.E., Benovic, J.L., Amlaiky, N., Unson, C., Milligan, G., Vinitsky, R., Spiegel, A.M. and Caron, M.G. (1987).** The D₂-dopamine receptor of anterior pituitary is functionally associated with a pertussis toxin-sensitive guanine nucleotide binding protein. *J. Biol. Chem.* 162, 4860-4867
- Seppala, M., Suikkari, A.M., and Julkunen, M. (1988).** Human endometrial protein. *Reprod. Nutr. Develop.* 28, 1649-1654
- Sharkey, N.A., Leach, K.L. and Blumberg, P.M. (1984).** Competitive inhibition by diacylglycerol of specific phorbol ester binding. *Proc. Natl. Acad. Sci. USA* 81, 607-610
- Shivers, B.D., Harlan, R.E., Hejtmancik, J.F., Comn, P.M. and Pfaff, D.W. (1986).** Localisation of cells containing LHRH-like mRNA in rat forebrain using in situ hybridization. *Endocrinology* 118, 883-885
- Singer, R.H., Lawrence, J.B. and Rashtchian (1987).** Chapter 4. Towards a rapid and sensitive in situ hybridization methodology using isotopic and nonisotopic probes. In: Valentino, K.L., Eberwine, J.H. and Barchas, J.D. (eds.), *In Situ Hybridization.* Oxford University Press, Oxford, P 70-94
- Sjoberg, J., Wahlstrom, T. and Seppala, M. (1984).** Pregnancy-associated plasma protein A in the human endometrium is dependent on the effect of progesterone. *J. Clin. Endocr. Metab.* 58, 359-362

- Stefanini, M., DeMartino, C. and Zamboni, L. (1967).** Fixation of ejaculated spermatozoa for electron microscopy. *Nature* **216**, 173-174
- Stray-Pedersen, S. (1982).** The effect of prolactin on fetal membrane transport. *J. Perinat. Med. (suppl 2)* **10**, 121-122
- Sutton, C.A. and Martin, T.F.J. (1982).** Thyrotropin-releasing hormone (TRH) selectively and rapidly stimulates phosphatidylinositol turnover in GH pituitary cells: A possible second step of TRH action. *Endocrinology* **110**, 1273-1280
- Taga, M., Satoh, M., Minaguchi, H., Mizuno, M. and Sakamoto, S. (1982).** Synthesis and secretion of prolactin in the decidualized human endometrium unassociated with pregnancy in vitro. *Endocr. Japan.* **29**, 335-340
- Taii, S., Ihara, Y. and Mori, T. (1984).** Identification of the mRNA coding for prolactin in the human decidua. *Bioch. Biophys. Res. Comm.* **124**, 530-537
- Takahashi, H., Nabeshima, Y., Nabeshima, Yo-Ichi, Ogata, K. and Takeuchi, S. (1984).** Molecular cloning and nucleotide sequence of DNA complementary to human decidual prolactin mRNA. *J. Biochem.* **95**, 1491-1499
- Takeda, A. and Leavitt, W.W. (1986).** Progestin-induced down regulation of nuclear estrogen receptor in uterine decidual cells: analysis of receptor synthesis and turnover by the density-shift method. *Biochem. Biophys. Res. Comm.* **135**, 98-104
- Tam, S.W. and Dannis, P.S. (1981).** The role of adenosine 3',5'-monophosphate in dopaminergic inhibition of prolactin release in anterior pituitary cells. *Endocrinology* **109**, 403-408
- Tamaya, T., Arabori, K. and Okada, H. (1985).** Relation between steroid receptor levels and prolactin level in the decidua of early human pregnancy. *Fertil. Steril.* **43**, 761-765
- Thraillkill, K. M., Golander, A., Underwood, L. E. and Handwerger, S. (1988).** Insulin-like growth factor I stimulates the synthesis and release of prolactin from human decidual cells. *Endocrinology* **123**, 2930-2934
- Thraillkill, K.M., Golander, A., Underwood, L.E., Richards, R.G., and Handwerger, S. (1989).** Insulin stimulates the synthesis and release of prolactin from human decidua cells. *Endocrinology* **124**, 3010-3014

- Tomita, K., MaCoshen, J. A., Fernandez, C. S. and Tyson, J. E. (1982a). Immunologic and biologic characteristics of human decidual prolactin. *Am. J. Obstet. Gynecol.* 142, 420-426
- Tomita, k., McCoshen, J. A., Friesen, H. G. and Tyson, J. E. (1982b). Quantitative comparison between biological and immunological activities of prolactin derived from human fetal and maternal sources. *J. Clin. Endocr. Metab.* 55, 269-271
- Toso, R.D., Sommer, B., Ewert, M., Herb, A., Pritchett, D.B., Bach, A., Shivers B. D. and Seeburg, P.H. (1989). The dopamine D2 receptor: two molecular forms generated by alternative splicing. *EMBO J.* 8, 4025-4034
- Tyson, J.E., Hawang, P., Guyda, H. and Friesen, H.G. (1972). Studies of prolactin secretion in human pregnancy. *Am. J. Obstet. Gynecol.* 113, 14-20
- Tseng, L. and Gurpide, E. (1975). Effects of progestins on estradiol receptor levels in human endometrium. *J. Clin. Endocr.. Metab.* 41, 402-404
- Tseng, L. and Gurpide, E. (1979). Stimulation of various 17- β and 20 α -hydroxysteroidhydrogenase activities by progestins in human endometrium. *Endocrinology* 104,1745-1748
- Tyson, J. E., Mowat, G. S. and McCoshen, J. A. (1984). Simulation of a probable biologic action of decidual prolactin on fetal membranes. *Am. J. Obstet. Gynecol.* 148, 296-300
- Tyson, J.E., McCoshen, J.A. and Dubin, N.H. (1985). Inhibition of fetal membrane prostaglandin production by prolactin: Relative importance in the initiation of labour. *Am. J. Obstet. Gynecol.* 151, 1032-1038
- Waites, G.T., Wood, P.L., Walker, R.A. and Bell, S.C. (1988). Immunohistological localization of human endometrial secretory protein, pregnancy-associated endometrial α 2-globulin (α 2-PEG), during the menstrual cycle. *J. Reprod. Fert.* 82, 665-672
- Walsh, S.W., Meyer, R.K., Wolf, R.C. and Friesen, H.G. (1977). Corpus luteum and fetoplacental functions in monkeys hypophysectomized during late pregnancy. *Endocrinology* 100, 845-850
- Wahlstrom, T. and Seppala, M. (1984). Placenta protein 12 (PP12) is induced in the endometrium by progesterone. *Fertil. Steril.* 41, 781-784
- Welshons, W. V., Lieberman, M.E. and Gorski, J. (1984). Nuclear localization of unoccupied oestrogen receptors. *Nature* 307, 747-749

- West, N.B. and Brenner, R.M. (1985).** Progesterone-mediated suppression of estradiol receptors in cynomolgus macaque cervix, endometrium and oviduct during sequential estradiol-progesterone treatment. *J. Steroid. Biochem.* **22**, 29-37
- West, N.B., McClellan, M.C., Sternfeld, M.D. and Brenner, R.M. (1987).** Immunocytochemistry versus binding assays of the estrogen receptor in the reproductive tract of spayed and hormone-treated macaques. *Endocrinology* **121**, 1789-1800
- Wetmur, J.G. (1975).** Acceleration of DNA renaturation rates. *Biopolymers* **14**, 2517-2524
- Wewer, U.M. Faber, M., Liotta, L.A. and Albrechtsen, R. (1985).** Immunochemical and ultrastructural assessment of the nature of the pericellular basement membrane of human decidual cells. *Lab. Invest.* **53**, 624-633
- Yamamoto, T., Nishiyama, M., Yanoh, K., Naka, Y., Naka, A. and Sugiyama, Y. (1989).** Regulation of human endometrial and decidual cell functions-role of epidermal growth factor in the decidualization. *Pro. Clin. Biol. Res.* **294**, 413-424
- Ying, Y.K., Kuslis, S., Maier, D.B., Riddick, D.H. and Randolph, J.F. (1988).** Stimulation of uterine prolactin secretion by human chorionic gonadotropin and progesterone in the cynomolgus monkey. *Fertil. Steril.* **50**, 976-979
- Zabavnik, J., Taylor, P.L., Peter, S.T. and Eidne K.A. (1991).** Expression of novel cloned rat pituitary receptors in mammalian cells. *Series H: Cell Biology* **52**, 101-113